

CAVEOLIN-1 AND ALTERED NEUREGULIN SIGNALING CONTRIBUTE TO THE
PATHOPHYSIOLOGICAL PROGRESSION OF DIABETIC PERIPHERAL NEUROPATHY

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Abstract

Objective: Diabetes is heterogeneous group of disorders characterized by aberrant insulin signaling and hyperglycemia. Chronic hyperglycemia leads to the development of complications including diabetic peripheral neuropathy (DPN). DPN is a pervasive complication associated with diabetes and its development is not completely understood.

Research Design and Methods: We aimed to determine if ErbB2 activation and the absence of Caveolin-1 (Cav-1) contribute to the development of DPN. We induced diabetes in Cav-1 knockout and wild-type mice and assessed thermal and mechanical sensitivity, motor and sensory nerve conduction velocity (MNCV/SNCV), ErbB2 activation, g-ratio, and epidermal nerve fiber density. The contribution of ErbB2 activation to DPN was evaluated using two ErbB2 inhibitors and a conditional double transgenic mouse line that expressed a constitutively active ErbB2 in myelinated Schwann cells.

Results: Diabetic mice exhibited decreased MNCV and thermal and mechanical sensitivity after acute diabetes, and these deficits were more severe and developed earlier in Cav-1 knockout mice. Hyperglycemia increased ErbB2 activity, which was amplified in Cav-1 knockout mice. Chronic hyperglycemia resulted in the additional development of reductions in SNCV and epidermal nerve fiber density. Treating diabetic mice with either ErbB2 inhibitor completely restored deficits after acute hyperglycemia and partially reduced deficits

after chronic hyperglycemia. Additionally, induction of constitutively active ErbB2 in myelinated Schwann cells in the absence of hyperglycemia was sufficient to induce a deficit in MNCV and mechanical sensitivity.

Conclusions: Cav-1 may be an endogenous regulator of ErbB2 activity, which when increased contributes to the pathophysiological development of diabetic peripheral neuropathy. Altered ErbB2 signaling is a novel mechanism that contributes to Schwann cell dysfunction in diabetes, and inhibiting ErbB2 may alleviate nerve dysfunction associated with diabetes.

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List of Abbreviations

3-DG	3-Deoxyglucosone
ADA	American Diabetes Association
AGE	Advanced Glycation End Product
ANS	Autonomic Nervous System
ARIA	Acetylcholine Receptor Inducing Activity
BDNF	Brain Derived Neurotrophic Factor
BMI	Body Mass Index
caErbB2	Constitutively Active ErbB2
CAN	Cardiac Autonomic Neuropathy
Cav-1	Caveolin-1
Cav-1 ^{-/-}	Caveolin-1 Knockout
CEL	Nε-(CarboxyEthyl) Lysine
CMAP	Compound Muscle Action Potential
CML	Nε-(CarboxyMethyl) Lysine
CNS	Central Nervous System
CNTF	Ciliary NeuroTrophic Factor
DAG	DiAcylGlycerol
DAN	Diabetic Autonomic Neuropathy
DCCT	Diabetes Control and Complications Trial
dnErbB4	Dominant Negative ErbB4
DPN	Diabetic PolyNeuropathy
DRG	Dorsal Root Ganglia
ED	Erectile Dysfunction
EGFR	Epidermal Growth Factor Receptor
ENFD	Epidermal Nerve Fiber Density
Erlotinib/Erlo	N-(3-ethylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine
GAD	Glutamic Acid Decarboxylase
GADA	Glutamic Acid Decarboxylase Antibodies

GAPDH	GlycerAldehyde-3-Phosphate DeHydrogenase
GGF	Glial Growth Factor
GI	GastroIntestinal
GPCR	G Protein Coupled Receptor
Hb A1C	Hemoglobin A1C
HLA	Human Leukocyte Antigen
HNF	Hepatocyte Nuclear Factor
IA-2A	Insulin Associated protein-2-Antibodies
IAA	Insulin AutoAntibodies
IAPP	Islet Amyloid PolyPeptide
ICA	Islet Cell Antibodies
IDDM	Insulin Dependent Diabetes Mellitus
IENF	IntraEpidermal Nerve Fiber
IFG	Impaired Fasting Glucose
IFN- γ	InterFeroN gamma
IGT	Impaired Glucose Tolerance
IL-1	InterLeukin-1
IL-10	InterLeukin-10
IL-6	InterLeukin-6
ip	IntraPeritoneal
LADA	Latent Autoimmune Diabetes in Adults
MAPK	Mitogen Activated Protein Kinase
MG	MethylGlyoxal
MHC	Major Histocompatibility Complex
MNCV	Motor Nerve Conduction Velocity
mRIPA	Modified RadioImmunoPrecipitation Assay
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCV	Nerve Conduction Velocity
NDF	Neu Differentiation Factor
NGF	Nerve Growth Factor
NIDDM	Non-Insulin Dependent Diabetes Mellitus

NO	Nitric Oxide
NRG-1	NeuReGulin-1
NRG-1	NeuReGulin-1
NRG-2	NeuReGulin-2
NRG-3	NeuReGulin-3
NRG-4	NeuReGulin-4
NT-3	NeuroTrophin-3
NT-4	NeuroTrophin-4
NT-5	NeuroTrophin-5
OCT	Optimal Cutting Temperature
OGTT	Oral Glucose Tolerance Test
PAI-1	Plasminogen Activator Inhibitor-1
PI3K	Phosphatidylinositol-3-phosphate Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PKI 166	4-(R)-phenethylamino-6-(hydroxyl)phenyl-7H-pyrrolo[2.3-d]-pyrimidine
PNS	Peripheral Nervous System
QST	Quantitative Sensory Test
RAGE	Receptor for Advanced Glycation End Products
rhNGF	Recombinant Human Nerve Growth Factor
ROS	Reactive Oxygen Species
rtTA	Reverse Tetracycline TransActivator Complex
SMDF	Sensory Motor Derived Factor
SNAP	Sensory Nerve Action Potential
SNCV	Sensory Nerve Conduction Velocity
STZ	Streptozotocin
SUR-1	SurfonylUrea Receptor-1
TCA Cycle	TriCarboxylic Acid Cycle
TNF- α	Tumor Necrosis Factor- α
TRE	Tetracycline Response Element

VEGF

Vascular Endothelial Growth Factor

WHO

World Health Organization

CHAPTER 1: DIABETES MELLITUS

1.1 Overview of Diabetes

Diabetes mellitus is a pervasive, heterogeneous group of disorders characterized by an increase in blood glucose arising as a result of deficits in insulin production, function, or both. In 2005 the World Health Organization (WHO) estimated that 220 million people worldwide suffered from diabetes mellitus (WHO 2009). The prevalence of diabetes mellitus has been increasing at an alarming rate for the past half century, and the number of people suffering from diabetes and its complications is expected to reach 300 million in the next 15 years (King, Aubert et al. 1998). In 2007 approximately 24 million people in the United States suffered from diabetes mellitus at a cost of \$174 billion (CDC 2007). Diabetes mellitus is costly in many aspects and attaining a better understanding of the disease, its complications, and the processes responsible for their development is critical in easing the suffering caused by these disorders.

The first recorded descriptions of diabetes depict a condition where the main symptom is polyuria, or excessive urination. Indeed, the word diabetes is derived from the Greek word *diabainein*, which roughly translated means “to pass or run through” as in a siphon (Engelhardt 1989; Eknayan and Nagy 2005). In the context of disease it means to discharge excessive amounts of urine, and thus, long before the causes of diabetes were determined, anyone presenting with polyuria was considered diabetic. Since that time several different types of diabetes have been identified and these fall into two main categories, diabetes mellitus and diabetes insipidus. Diabetes insipidus results from the lack of production of vasopressin or the inability of nephrons to utilize vasopressin (Baylis and

Cheetham 1998). The absence of vasopressin results in the body's inability to retain water, resulting in polyuria, or excessive urination (Baylis and Cheetham 1998). This work will focus on diabetes mellitus and therefore no subsequent discussion of diabetes insipidus will take place. Additionally, diabetes and diabetes mellitus will be used interchangeably throughout this dissertation.

Diabetes mellitus is a group of several disorders that includes type 1 diabetes, type 2 diabetes, gestational diabetes, and secondary diabetes. In the United States and throughout the world, about 10% of all diabetes cases are type 1 diabetes, while type 2 diabetes comprises approximately 90% of all diabetes (WHO 2009). Gestational diabetes and other types of diabetes account for approximately 1% of diabetes cases (CDC 2007). Each type of diabetes has distinctive epidemiologic characteristics and can be distinguished from the others by its etiology.

Type 1, type 2, and gestational diabetes result in an increase in blood glucose levels, but the root causes of an increase in blood glucose differentiate specific types of diabetes. Type 1 diabetes, previously referred to as juvenile-onset diabetes and insulin dependent diabetes mellitus (IDDM), results from the autoimmune mediated destruction of the insulin producing beta cells in the pancreas. The cause of type 2 diabetes, previously referred to as adult-onset and non-insulin independent diabetes mellitus (NIDDM), is not completely understood. During the development of type 2 diabetes mellitus, cells become resistant to the action of insulin, no longer take up glucose and after time the pancreas loses its ability to synthesize and secrete insulin. Type 2 diabetes is associated with an older age of onset, obesity, physical inactivity, and race, where people of African, Hispanic, or native American descent are at a higher risk than other populations (CDC 2007). Gestational diabetes arises

as a result of increased placental hormone production and the development of insulin resistance. Since placental hormone production is proportional to placental size, gestational diabetes generally develops in the second or third trimester and manifests in a fashion similar to that of type 2 diabetes (Catalano, Huston et al. 1999). Since gestational diabetes manifests in a manner resembling type 2 diabetes, the discussion of type 2 diabetes will also apply to gestational diabetes. Diabetes can arise from other causes as well, including pancreatic disease, production of counterregulatory hormones, abnormal insulin, dysfunctional insulin receptors, production of antibodies targeting the insulin receptor, or by chemicals that selectively target and destroy insulin producing β -cells in the pancreas (Inzucchi 2003). Diabetes caused by one of these things is often referred to as secondary diabetes. Each type of diabetes arises because of specific cellular events and progresses in a unique manner. Thus, the classification of diabetes depends on these factors. However, this has not always been the case. The classification, cause, and diagnosis of the group of diseases we now call diabetes have evolved since the condition was first described a few millennia ago.

1.2 The History of Diabetes

The group of diseases we now refer to as diabetes were first observed by the Egyptians about 3500 years ago. The Eber's Papyrus describes a condition known as the sugar disease, which was characterized by polyuria (Ahmed 2002; King and Rubin 2003). The term diabetes is often attributed to Demetrius of Apameia who, in the 2nd century, introduced the word *diabainein*. Recall from an earlier discussion that in Greek *diabainein* means to pass or run through as in a siphon (Engelhardt 1989; Eknayan and Nagy 2005). Shortly after Demetrius coined the word diabetes, fellow Greek, Aretaeus used the new term to describe what many consider the first clinical description of diabetes. He described

diabetes as a rare condition of the bladder and kidneys where the flesh and limbs were transformed into urine (Engelhardt 1989; Ahmed 2002; King and Rubin 2003; Eknayan and Nagy 2005). A contemporary of Aretaeus, Galen, having determined that the kidneys attracted the “watery substance” from blood and were therefore the source of urine, concluded that diabetes was a disease of the kidneys alone (Eknayan and Nagy 2005). Additionally, some time between 1000 BC and the 2nd century, Indian texts, including those of Susruta, describe a disease characterized by polyuria, where the urine is described as *ksaudra* (sweet) or like *madhu* (honey), more common among rich people who consumed large quantities of food (King and Rubin 2003; Eknayan and Nagy 2005).

In India, diabetics were often identified by the greater number of ants and wasps near their chamberpots. However, the numerous observations that diabetes resulted in sweet urine did not have an impact on medical advancement of the disease until Thomas Willis made the observation in 1674 (Engelhardt 1989; King and Rubin 2003; Eknayan and Nagy 2005). When Willis made the observation that urine from a diabetic patient was sweet, the Latin word *mellitus*, meaning honey, was appended to diabetes. Furthermore, Willis argued for the first time that the cause of diabetes inhabited the blood not the kidneys, asserting that any sugar in the urine had to originate in the blood (Engelhardt 1989; Eknayan and Nagy 2005). A century later, Mathew Dobson confirmed Willis’ assertion that the excess sugar in the urine of diabetics originated in the blood, by accurately quantifying the sugar content of the blood in diabetic patients for the first time (King and Rubin 2003; Eknayan and Nagy 2005). At this time, due to the observation that some diabetics did not have sweet urine, the distinction between diabetes mellitus and diabetes insipidus is made. In 1815 the sugar in

diabetic urine was determined to be glucose, but there was still uncertainty as to whether the disease resided in the blood or kidneys (Eknoyan and Nagy 2005).

In 1889, Oscar Minkowski and Joseph Mering confirmed Thomas Cawley's suggestion that the pancreas was involved in diabetes when they observed the development of diabetes in pancreatectomized dogs (Engelhardt 1989; King and Rubin 2003; Eknoyan and Nagy 2005). Medical student Paul Langerhans' study and description of the pancreas led Edward Sharpey-Shafer to suggest in 1916 that the islets of Langerhans produced a glucose-regulating hormone, which he called insulin. Shortly thereafter, Frederick Banting and Charles Best, working in John J. R. MacLeod's lab, extracted the hormone insulin from healthy dogs and injected it into pancreatectomized dogs (Engelhardt 1989; King and Rubin 2003; Eknoyan and Nagy 2005). After seeing the transient benefits of insulin in dogs, Banting and Best sought the help of biochemist James Collip, who was able to purify insulin from cattle and successfully treat human patients with diabetes mellitus for the first time in history (King and Rubin 2003). The importance of their discovery was rewarded in 1923 when Banting and MacLeod received the Nobel Prize in physiology or medicine (nobelprize.org 2010).

After Banting and Best, diabetes related research focused on improving the purification and efficacy of insulin, other treatment options, and complications associated with diabetes. Working from the structure of insulin discovered by Frederick Sanger in the 1950's, Panayotis Katsoyannis and Helmut Zahn produced synthetic insulin, which became the main source of insulin for the treatment of diabetes (Sanger 1950; Brown, Sanger et al. 1955; Harris, Naughton et al. 1956; Katsoyannis 1964). In 1975 the first prototype of a continuous subcutaneous insulin infusion device, known today as an insulin pump, was

introduced (Thomas and Bessman 1975). Five years later, the first trial using recombinantly derived human insulin took place, and since 1982 recombinant human insulin has been the main source of insulin for diabetic patients (Keen, Glynne et al. 1980). Since the 1960's, islet cell transplantation has also been an area of active research. To date, approximately 400 people have received islet cell transplants, with all patients demonstrating improved blood glucose control and 10% remaining insulin-independent after five years (Alejandro, Barton et al. 2008). A great deal has been learned about diabetes in the past, and current diabetes research focuses on unraveling the etiology of the complications associated with diabetes, preventing diabetes, and refining our understanding of what causes both types of diabetes (NIDDK 2003).

1.3 Diagnosis and Classification of Diabetes

The American Diabetes Association (ADA) and the World Health Organization (WHO) define diabetes mellitus as a group of metabolic disorders characterized by hyperglycemia resulting from deficits in insulin secretion, action, or both. Over the years, requirements for a diagnosis of diabetes have changed as populations and technology have changed, and current diagnoses are made using one of several criteria described in Table 1.1 (Malchoff 1991; 2006; WHO 2009).

Table 1.1: Diagnostic Criteria for Diabetes Mellitus

1. Presentation of symptoms and casual blood glucose of ≥ 200 mg/dL
2. Fasting plasma glucose of ≥ 126 mg/dL
3. 2-hour post load glucose level ≥ 200 mg/dL during an oral glucose tolerance test (OGTT), containing a glucose load of 75 g anhydrous glucose dissolved in water
4. Glycated hemoglobin levels $> 6.5\%$

Casual blood glucose is defined as any time of day without regard to time of last meal. Fasting is defined as zero caloric intake for at least eight hours (2006; 2009; WHO 2009).

Common symptoms of diabetes include polyuria, glucosuria (sugar in the urine), polydipsia (excessive thirst), unexplained weight loss, growth impairment, and susceptibility to infections. When these symptoms are observed, one of the requirements in Table 1 must be met on the date of initial testing and again on a different day in order for a diagnosis of diabetes to be conferred (2006; WHO 2009). The World Health Organization suggests the use of fasting plasma glucose measurement as the standard method for the diagnosis of diabetes. The WHO also recommends using an OGTT only as a secondary means of establishing a diabetes diagnosis (WHO 2006). A more recently developed tool used to diagnose diabetes is the measurement of glycated hemoglobin (Hb A1C) levels. Only recently approved for use by an international committee, though not recommended by the WHO, Hb A1C levels can be used to estimate average glycemic status of the previous two to three months (Koenig, Peterson et al. 1976; Koenig, Peterson et al. 1976; 2006; WHO 2006; 2009; 2009). According to Koenig and colleagues, Hb A1C levels best reflect blood glycemic status from the previous week to month and that periodic Hb A1C measurement offers a method to evaluate recent glycemic control (Koenig, Peterson et al. 1976; Koenig, Peterson et al. 1976). One of the original problems cited with Hb A1C measurement was the absence of standardization for testing methods and procedures. An international expert committee compiled by the ADA recently concluded that current methods for measuring Hb A1C are highly standardized and therefore recognized as a valid method for diagnosing diabetes (2009; 2009). The methodologies described above are used to diagnose type 1 and type 2 diabetes, but they are also used to identify people at risk of developing diabetes.

In some cases, a person presents with blood glucose values that are elevated, but not so much so that they would be diagnosed as diabetic. The ADA and the WHO both define

this intermediate condition as impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) (2006; WHO 2006). Traditionally, IGT is used when an oral glucose tolerance test is performed to establish the diagnosis, and corresponds with a two-hour post-load glucose level of 140-199 mg/dL. IFG is used when the fasting blood glucose method is employed for diagnosis and corresponds with a fasting blood glucose level of 100-125 mg/dL (2006; WHO 2006). Even though there is evidence for a subtle distinction between IGT and IFG, patients with one of these conditions, more commonly referred to as pre-diabetes, are at a higher risk for developing diabetes (Inzucchi 2003). Persons with OGTT and fasting plasma glucose levels higher than those just mentioned would be past the pre-diabetes stage and would be diagnosed as having diabetes.

1.3.a Type 1 Diabetes Mellitus

Type 1 diabetes was formerly referred to as juvenile onset diabetes and insulin dependent diabetes mellitus (IDDM), but is now referred to as type 1 diabetes in order to more accurately reflect the underlying pathophysiology of the disorder (Inzucchi 2003). Type 1 diabetes is characterized by the autoimmune mediated destruction of insulin producing pancreatic β -cells and invariably requires insulin therapy. Type 1 diabetes that occurs in the absence of autoantibodies is known as idiopathic diabetes and is strongly inherited in a very small portion of type 1 diabetic patients (2006). As mentioned previously, type 1 diabetes comprises between 5-10% of all diabetes diagnoses and the incidence of type 1 diabetes is projected to remain stable (Winer and Sowers 2004; 2006; WHO 2009). Estimates of the prevalence of type 1 diabetes in the United States range from 1.25 million to 2.5 million, with an approximate incidence of 160,000 new cases each year (CDC 2007). Studies on the prevalence and incidence in non-Caucasian populations are limited, but non-

Caucasian children are less likely to have the presence of autoantibodies, though there is no difference in the number of children that develop type 1 diabetes between Caucasian and non-Caucasian groups (Bennett 2003). Furthermore, there does not seem to be a difference in the prevalence or incidence of type 1 diabetes among males and females (Bennett 2003). Type 1 diabetes usually develops early in childhood and occurs as a result of autoantibody production and β -cell destruction. However, it may develop around mid-life and is called latent autoimmune diabetes in adults (LADA) (Naik 2003; 2006). The natural history of diabetes contains four stages, starting with the development of autoimmunity and then the onset of hyperglycemia followed by a short period of remission, then the return and persistence of hyperglycemia, and finally the development of diabetic complications.

For a long time it was thought that the pre-clinical stage of diabetes was relatively short, and that once the process began, chronic hyperglycemia was not far behind. We now know that the disease process is active for several years in most cases, before chronic hyperglycemia develops. The initial stage in the development of type 1 diabetes mellitus is the production of diabetes-associated autoantibodies. Four main diabetes-associated autoantibodies have been described and include islet cell antibodies (ICA), insulin autoantibodies (IAA), glutamic acid decarboxylase antibodies (GADA), and insulin-associated protein-2 antibodies (IA-2A) (Schatz, Krischer et al. 1994; Ziegler, Hummel et al. 1999; Naik 2003). The first and most frequent autoantibody to appear is usually high affinity IAA, although GADA can be expressed concomitantly or shortly after IAA expression (Ziegler, Hummel et al. 1999). However, the presence of any single autoantibody is not a good predictor of diabetes development. Not all patients with autoantibodies develop diabetes, and autoantibodies appear in a small portion of the general population as well (Naik

2003). In contrast, the presence of one or more antibodies is a good predictor for the eventual development of diabetes. Children that tested positive for one or more autoantibodies were more likely to develop diabetes, and the more antibodies expressed translated into an even greater risk and earlier onset of diabetes (Ziegler, Hummel et al. 1999; Naik 2003).

There is no doubt that β -cell destruction accounts for a considerable portion of the decrease in insulin production that signifies preclinical diabetes, but there is additional evidence that cytokines, genetics, and other factors may contribute to the impairment in insulin secretion observed in type 1 diabetes. Indeed, at least 80-90% of the functional activity of β -cells must be destroyed before persistent hyperglycemia develops (Naik 2003). It is important to gain an understanding of how these factors contribute to the production of autoantibodies in order to comprehend how type 1 diabetes develops.

Cytokines modulate and help direct the immune response but can also be directly toxic to β -cells. The cytokine interferon gamma ($\text{IFN-}\gamma$) has been shown to increase expression of major histocompatibility complex molecules (MHC) while interleukin-1 (IL-1) inhibits glutamic acid decarboxylase (GAD) (Naik 2003). GAD catalyzes the decarboxylation of glutamic acid to produce gamma-amino butyric acid, but how the inhibition of GAD contributes to the development of diabetes is not clear. The cytokine interleukin-10 (IL-10) and other cytokines released by T-helper cells also accelerate β -cell destruction (Naik 2003). Furthermore, it appears that expression of multiple cytokines increases their toxic affects. There is also evidence to suggest that cytokines inhibit insulin secretion, thereby increasing the immune system's potency in stopping insulin production (Naik 2003).

The human leukocyte antigen (HLA) system is the name of the MHC in humans and plays a crucial role in the function of the immune system. Major histocompatibility complexes are responsible for presenting antigens so that the immune system can determine if that cell is a self or alien cell. Since type 1 diabetes most commonly results from immune mediated destruction of β -cells, genetic differences in the HLA can have a large impact on the development of diabetes. Additionally, the HLA system contains massive numbers of polymorphisms (Svejgaard 1982). The full gamut of type 1 diabetes related HLA haplotypes and genotypes is beyond the scope of this work. However, the most prevalent HLA factors observed in type 1 diabetes will be briefly covered. Initial genetic forays looking at HLA and diabetes showed that HL-A8 and HL-W15 were potential genetic markers for type 1 diabetes since these factors are involved in altered T-lymphocyte response (Nerup 1982). Other studies found an extremely significant association ($p < 0.00000000001$) between HLA-B8 and HLA-B15 positivity and type 1 diabetes (Nerup 1982; Naik 2003). Later studies though, found that HL-DR3, and HL-DR4 alleles conferred a greater risk of developing type 1 diabetes. Indeed, diabetic patients with HLA-B8 also contain HL-DR3 more often than HLA-B8 positive non-diabetics (Naik 2003). Furthermore, 90-95% of all type 1 diabetic patients are HL-DR3 or DR4 positive, while this occurs in approximately 5-10% of non-diabetics (Nerup 1982; Naik 2003). Certain other HLA factors confer an even higher risk of developing type 1 diabetes. These include the HLA-DQ alleles, certain combinations of which modulate the severity of the risk (Naik 2003). It remains to be explained why these factors put individuals at a higher risk for developing type 1 diabetes, but the current thought is that these alleles result in the modification of class II MHC molecule expression.

Cytokines and genetic factors contribute to the initial development of autoantibodies, which specify the first pre-clinical stage of type 1 diabetes that is followed by the presence of additional autoantibodies. IAAs are usually the first autoantibodies to develop. One to two years after the initial observance of IAA autoantibodies, a second and third autoantibody, usually GADA and IA-2A are present (Ziegler, Hummel et al. 1999). Occasionally, multiple autoantibodies appear within a year of initial autoantibody presence, but more significantly, by the time individuals develop hyperglycemia they almost invariably have all four major autoantibodies (Ziegler, Hummel et al. 1999). Aside from an increase in the types of antibodies present, epitope spreading within individual antibodies also occurs and contributes to the destruction of β -cells and islet proteins. As early as 1988, Schatz and colleagues observed the polyclonal nature of islet autoantibodies (Schatz, Barrett et al. 1988). Epitope spreading, or maturation as it is often called, has been studied most extensively in GADAs and occurs for an extended period of time before the onset of sustained hyperglycemia (Bonifacio, Lampasona et al. 2000; Naik 2003). Bonifacio and colleagues observed that primary GADAs targeted the middle portion, residues 96-444, of GAD (Bonifacio, Lampasona et al. 2000). Subsequent analysis showed antibody reactivity to the amino terminus, and in limited cases the carboxy terminus, of GAD (Bonifacio, Lampasona et al. 2000). As epitope spreading continues, insulin production gradually wanes until hyperglycemia is inevitable.

Once enough autoantibody mediated β -cell destruction has occurred, insufficient amounts of insulin are secreted and hyperglycemia ensues. Upon the onset of hyperglycemia, insulin treatment is almost always prescribed. Initiation of insulin treatment generally leads to a phenomenon where insulin dose has to be reduced or eliminated for a

short period of time. This phenomenon is known as the honeymoon period or transient partial remission of the disease. Remission is most likely due to temporary recovery of β -cell function and increased insulin secretion (Akirav, Kushner et al. 2008; Mortensen, Hougaard et al. 2009). Currently, the condition is medically defined by its treatment, although it has been suggested that the condition should be defined by the recovery of β -cell function (Mortensen, Hougaard et al. 2009). Unfortunately, the period of remission is short-lived, hyperglycemia returns, and the development of complications follows.

The development of complications marks the fourth and final stage of the development of diabetes and correlates with duration of disease and degree of glycemic control. At the onset of hyperglycemia there is little evidence for the occurrence of clinical complications. After this lag period though, the incidence of clinical complications rises dramatically. Close to 50% of type 1 diabetics have retinopathy after seven years of diabetes and this figure increases to greater than 90% after seventeen years (Palmberg, Smith et al. 1981). Few diabetic patients demonstrate preclinical signs of nephropathy upon being diagnosed, but do demonstrate increased albumin excretion rates, a predictor of future development of nephropathy, after five years (Steinke, Sinaiko et al. 2005). Hyperglycemia seems to impact small nerve fibers mediating temperature discrimination early on whereas longer durations of hyperglycemia influence both small and large fibers, producing a neuropathy that encompasses more fiber types (1988; Ziegler, Mayer et al. 1988; Valensi, Giroux et al. 1997). Within a year of diagnosis less than 10% of patients demonstrate evidence of cardiovascular disease. After 12 years the incidence increases to about 15% and after 30 years it approaches nearly 20% (Orchard, Olson et al. 2003; Pambianco, Costacou et al. 2006). The incidence of cardiovascular disease may be small compared to other

complications, but it accounts for almost 70% of diabetes associated deaths in the United States (CDC 2007). The development of these complications is tightly linked to glycemic control. Maintaining near normal blood glucose levels is the best way to slow the progression of diabetes related complications (1995; 1995). Maintaining near normal blood glucose levels over extended periods of time though, can be difficult in the presence of the extensive β -cell loss that causes type 1 diabetes.

1.3.b Type 2 Diabetes Mellitus

Just as the nomenclature for type 1 diabetes has undergone recent changes that more accurately reflect the underlying pathophysiology, so has the nomenclature for type 2 diabetes mellitus. Type 2 diabetes was formerly referred to as adult onset diabetes mellitus and non-insulin-dependent diabetes mellitus (NIDDM) (CDC 2007; WHO 2009). Type 2 diabetes is characterized by the development of insulin resistance in muscle, adipose, and liver tissue, which eventually transforms into β -cell dysfunction, deficits in insulin secretion, and hyperglycemia (Inzucchi 2003; 2006; WHO 2006). Type 2 diabetes accounts for about 90-95% of all cases of diabetes mellitus and is generally diagnosed in patients older than forty. However, the average age at which type 2 diabetes is being diagnosed is decreasing (King, Aubert et al. 1998; 2000; Fagot-Campagna, Narayan et al. 2001; 2006; CDC 2007; WHO 2009). More children and individuals under twenty years of age are being diagnosed with type 2 diabetes today than ever before (CDC 2007; WHO 2009). The incidence of type 2 diabetes in the United States is approximately 1.5 million new cases each year, with a disproportionate number of diagnoses occurring among ethnic minority groups (CDC 2007). Additionally, there is a slight difference in the prevalence and incidence of type 2 diabetes between sexes. Type 2 diabetes occurs more in women throughout the world than it does in

men, although economic stability of a region has an influence on differences in diagnoses between the sexes (King, Aubert et al. 1998).

Lifestyle choices have a significant impact on whether type 2 diabetes develops early or later in life. Long before diabetes was even given a name, the condition was associated with the wealthy people who tended to consume large quantities food and work less than other people (Eknoyan and Nagy 2005). The World Health Organization attributes the development of type 2 diabetes to excess body weight and physical inactivity (WHO 2009). Additional studies have shown that body mass index (BMI) and the development of diabetes are directly proportional. That is, as BMI increases, so do the chances of developing insulin resistance and type 2 diabetes (Must, Spadano et al. 1999; Hu, Manson et al. 2001). In some cases BMI is the greatest predictor of progression toward diabetes (Hu, Manson et al. 2001). Furthermore, diet, and physical inactivity are also associated with the development of type 2 diabetes. People with poor diets and less than thirty minutes of physical activity each week were almost guaranteed to develop diabetes, while people getting more exercise were less likely to develop type 2 diabetes mellitus (Hu, Manson et al. 2001; Kelley and Goodpaster 2001). Additionally, there is a correlation between exercise and mortality in diabetes, such that men with diabetes who get regular exercise are at a lower risk of mortality (Church, Cheng et al. 2004).

Early studies looking at the natural history of type 2 diabetes concluded that patients undergo a period of IFG or IGT before developing hyperglycemia and type 2 diabetes (Edelstein, Knowler et al. 1997). As insightful as these studies were, the question of whether or not the development of type 2 diabetes required the transition from normoglycemia to IFG and or IGT remained unanswered. Additionally, the timeline for the

transition from normoglycemia to IFG/IGT to hyperglycemia had not been defined. Meigs and associates measured fasting plasma glucose and plasma glucose two hours after glucose load about every two years for twenty years and found that ten times as many subjects developed IGT than IFG (Meigs, Muller et al. 2003). Furthermore, they found that after five years over a third of subjects developed IGT while only 2.5% developed diabetes (Meigs, Muller et al. 2003). By 20 years, 71% had developed IGT and approximately 21% developed type 2 diabetes (Meigs, Muller et al. 2003). From this study, it is evident that the development of type 2 diabetes does not follow a defined path from normoglycemia to IGT to IFG to type 2 diabetes. Instead, the progression from normoglycemia to type 2 diabetes is quite variable and most often includes the development of IGT, but can include the development of IGT with subsequent development of IFG or the development of IFG alone.

Regardless of the path type 2 diabetes takes to develop, there are several key factors that promote the progression from normoglycemia to IGT/IFG to chronic hyperglycemia: β -cell dysfunction, insulin resistance, and genetic factors. Normally, insulin secretion follows a pulsatile and oscillatory pattern where release volume oscillates on a 120-minute cycle with pulses of insulin being secreted about every ten to fifteen minutes (Kahn 2003). Not only do type 2 diabetics demonstrate dysfunctions in the pattern of insulin release, but the volume of insulin being released is altered as well. When plasma glucose levels are accounted for, patients with type 2 diabetes demonstrate impaired basal release of insulin (Turner, McCarthy et al. 1976; Halter, Graf et al. 1979). Similar deficits are observed for glucose-stimulated insulin release. Following glucose challenge, patients with IGT and type 2 diabetes demonstrate a significant deficit in β -cell function evidenced by a decrease in insulin release (Lillioja, Mott et al. 1993; Chen, Boyko et al. 1995; Kahn 2003). That is, β -

cell dysfunction increases with deteriorating glucose tolerance. β -cell dysfunction and impaired insulin release are most notable immediately following glucose challenge, during the first phase of the insulin response (Kahn 2003). Second phase insulin release is also impaired in IGT and type 2 diabetes, though not to the same degree as first phase release. The reasons for these deficits are not completely understood. Although a definitive explanation remains to be determined, studies suggest that a loss of β -cell number, a decrease in β -cell mass, or some combination of the two contribute to impaired steady state and glucose stimulated insulin release.

β -cell dysfunction is not the only factor contributing to the development of type 2 diabetes. Another hallmark of type 2 diabetes mellitus is insulin resistance. Insulin resistance is defined as a state in which a given concentration of insulin produces a less than normal biological response (Olefsky 2003). The development of insulin resistance generally precedes the development of type 2 diabetes and is caused by the secretion of abnormal insulin, insulin antagonists, and defects in target tissues.

One of the factors contributing to the development of insulin resistance is the secretion of structurally abnormal insulin. Several studies have reported the presence of structurally aberrant insulin from β -cells within the pancreas (Rabinowitz and Zierler 1962; Tager, Given et al. 1979; Given, Mako et al. 1980). Irregular cleavage of proinsulin to insulin, caused by structural abnormalities at the cleavage site, can also lead to the formation of structurally defective insulin (Olefsky 2003). The secretion of such insulin leads to insulin resistance because target tissues cannot receive and respond to the insulin. Fortunately, such patients respond favorably to exogenous insulin (Olefsky 2003).

Patients with insulin antagonists circulating in the blood however, would not respond positively to exogenous insulin therapy. Insulin antagonists generally fall into one of two categories, hormonal antagonists and non-hormonal antagonists. Hormonal antagonists are most often counterregulatory hormones such as glucocorticoids, growth hormone, glucagon and the catecholamines (Olefsky 2003). Non-hormonal antagonists which are commonly found circulating in the blood of type 2 diabetic patients include free fatty acids, which are thought to alter glucose usage and therefore insulin function, anti-insulin receptor antibodies, anti-insulin antibodies, and resistin (Olefsky 2003). Resistin is secreted by adipocytes and has been shown to antagonize the cellular effects of insulin (Steppan, Bailey et al. 2001). Islet amyloid polypeptide (IAPP) and cytokines have also been linked to insulin resistance (Olefsky 2003). These numerous insulin antagonists contribute to the development of insulin resistance, but they are not the only contributing factors.

The last factor contributing to the development of insulin resistance is the defects in target tissues and insulin receptors. Insulin binding to the α -subunit of the insulin receptor leads to activation of the tyrosine kinase activity of the β -subunit and subsequent autophosphorylation of the receptor. Autophosphorylation leads to activation of insulin receptor substrate proteins and ultimately the initiation of a signaling cascade, which mediates insulin's many biological actions. Since there are several steps in the transduction of insulin to activation of a signaling cascade, any abnormality in this process has the potential to produce insulin resistance. Indeed, Olefsky and Reaven reported that insulin binding to the insulin receptor was significantly decreased in type 2 diabetic patients (Olefsky and Reaven 1977). The autophosphorylation of the insulin receptor that is critical for eliciting insulin's biological actions is also deficient in type 2 diabetes (Freidenberg,

Henry et al. 1987). A defect in any portion of the insulin signaling pathway can result in insulin resistance.

Although obesity and physical inactivity are known to be risk factors for developing insulin resistance and type 2 diabetes, these factors alone are insufficient in most cases to cause disease. The interaction of the environment with insulin resistance and preexisting genetic factors, related to β -cell dysfunction, also contribute to the development of type 2 diabetes mellitus. Several genes have been proposed to be involved in the development of type 2 diabetes and include glucokinase, the high affinity β -cell sulfonylurea receptor (SUR-1), the glucose transporter GLUT-2, the glucagon receptor, and hepatocyte nuclear factor (HNF) (Kahn 2003). Although polymorphisms in the coding region of the glucokinase gene have not been found, polymorphisms in the promoter region of this gene were found to be associated with impaired glucose tolerance and reduced insulin response (Stone, Kahn et al. 1996). SUR-1 plays a role in coupling glucose metabolism to insulin secretion and variants in the SUR-1 gene at exons twenty-two and twenty-four were more common in type 2 diabetic patients than in the general population (Kahn 2003). This association is, however, only observed in some populations of type 2 diabetics. Another gene that plays a role in glucose sensing and insulin secretion is the low affinity glucose transporter GLUT-2, which is predominantly expressed in pancreatic β -cells (Kahn 2003). Mutations in GLUT-2 lead to impaired glucose response, but have not been linked to type 2 diabetes mellitus (Janssen, Bogardus et al. 1994; Matsubara, Tanizawa et al. 1995). A missense mutation in the glucagon receptor associated with the IGT and type 2 diabetes has also been reported (Hager, Hansen et al. 1995). The association between the mutated receptor and IGT and type 2 diabetes mellitus is likely caused by glucagon having a significantly lower affinity for the

mutant receptor (Kahn 2003). The increase in type 2 diabetes incidence among younger populations led investigators to look for genetic factors that might be involved in the development of type 2 diabetes in such populations. Mutations in hepatocyte nuclear factor-4 α , -1 β , and -1 α (HNF-4 α , HNF-1 β , and HNF-1 α) have all been shown to be associated with an earlier onset of type 2 diabetes in some populations (Kahn 2003). Not all populations of type 2 diabetic patients contain all of the polymorphisms associated with the disease, but the polymorphisms discussed occur in a sufficient number of type 2 diabetics to be considered contributing factors in the development of insulin resistance.

The development of type 2 diabetes is quite polygenic in nature and the development of chronic hyperglycemia requires an interplay among obesity, physical inactivity, and genetic factors that promote β -cell dysfunction and insulin resistance. The right combination of these factors promotes the development of chronic hyperglycemia that is distinctive of type 2 diabetes. As with type 1 diabetes mellitus, once hyperglycemia sets in, the development of complications follows. Not all diabetic patients will develop each of these complications, but most will develop at least one of the complications described below.

1.3.c Complications of Diabetes

Hyperglycemia is the distinguishing characteristic of type 1 and type 2 diabetes and results in a plethora of complications in many organ systems. Complications arising from type 1 diabetes and type 2 diabetes can be microvascular or macrovascular in nature and include cardiovascular disease, cognitive dysfunction, nephropathy, neuropathy, peripheral vascular disease, retinopathy, and stroke (WHO 1985; Biessels, Kappelle et al. 1994; Bennett 2003; Manschot, Brands et al. 2006; CDC 2007; WHO 2009).

Vascular Disease

The diabetic complication with the greatest impact on mortality and morbidity is cardiovascular disease, which accounts for 50% of deaths associated with diabetes (Young 2003; CDC 2007; WHO 2009). The main components of cardiovascular disease are coronary artery disease and hypertension. Cardiovascular disease generally manifests as angina, acute myocardial infarction, heart failure, or sudden death (Young 2003).

Atherosclerotic coronary artery disease is the most common cause of heart failure in diabetic patients (Kannel, Hjortland et al. 1974). Some of the atherosclerosis observed in diabetes can be attributed to dyslipidemia, correlating with a decrease in high-density lipoprotein, an increase in triglycerides, and increased oxidative modification of low-density lipoprotein (Semenkovich 2003). Hypertension on the other hand, occurs in 40-60% of diabetic patients and often exacerbates other diabetes associated complications (Kaplan 2003; Young 2003). Hypertension associated with type 2 diabetes is primarily thought to be a product of obesity and inactivity (Kaplan 2003).

Diabetic patients are also prone to peripheral vascular disease, or a decrease in perfusion of the blood vessels in the periphery. The prevalence of peripheral vascular disease increases from about 10% in patients recently diagnosed with diabetes to almost 40% in patients with more than twenty years of diabetes (Melton, Macken et al. 1980; Palumbo 1995). The development of peripheral vascular disease is considered to be a result of hyperglycemia induced changes in the basement membrane, smooth muscle cells, and endothelial cells of blood vessels (Akbari 2003). These changes lead to functional impairment of blood flow in many blood vessels throughout the body. One of the most obvious consequences of blood vessel dysfunction is in what is commonly referred to as diabetic foot disease. Neuropathy, infection, and ischemia contribute to the development of

tissue necrosis, ulceration, and gangrene often seen in the feet of diabetic patients (Akbari and LoGerfo 1999). Without proper perfusion, what would normally be minor lacerations or abrasions becoming major medical problems for people with diabetes. Decreased perfusion also has a significant impact on blood flow in cerebrum and is a major risk factor for ischemic stroke.

Stroke

Another complication with a significant impact on mortality is stroke. Approximately 45% of all strokes can be attributed to diabetes and people with diabetes are about two to four times more likely than non-diabetics to suffer a stroke (CDC 2007; WHO 2009). Diabetic patients with other complications are also at a greater risk for suffering ischemic stroke (Akbari 2003). Furthermore, strokes are generally worse when occurring in diabetic patients than in non-diabetic patients (Jorgensen, Nakayama et al. 1994; Kissela, Khoury et al. 2005). One of the reasons diabetic patients are at a greater risk for ischemic stroke is because hyperglycemia induces morphologic abnormalities in cerebral blood vessels. These changes include arterial endothelial cell necrosis, capillary basement membrane thickening, and decreased endothelial-mediated vasodilation (Hogikyan, Galecki et al. 1998; Akbari 2003). There is also evidence to suggest that coagulation may be affected by diabetes. Study participants that demonstrated insulin resistance also demonstrated an increased susceptibility for blood coagulation, which would increase the likelihood of stroke in already compromised blood vessels (Romano, Guagnano et al. 2003). As noted earlier, diabetes increases the development of atherosclerosis and hypertension. Both of these events also increase the risk of stroke. Higher blood pressure in weakened and thickened blood vessels makes them more susceptible to occlusion and rupture. Although few studies have focused

on stroke in diabetic patients alone, diabetic patients treated with antihypertensive medication demonstrated a 73% reduction in stroke occurrence, compared to 38% in non-diabetic patients (Tuomilehto, Rastenyte et al. 1999). Diabetes does not cause stroke directly, but the microvascular and macrovascular changes initiated by chronic hyperglycemia result in a drastically increased propensity for stroke.

Nephropathy

Diabetic nephropathy is the leading cause of chronic kidney disease and is characterized by proteinuria, or the presence of protein in the urine. Diabetic nephropathy, like all diabetic complications, is associated with disease duration and affects between 30-40% of diabetic patients with disease duration of greater than twenty years (Krolewski, Warram et al. 1987; Ballard, Humphrey et al. 1988; Krolewski, Eggers et al. 1996; CDC 2007; WHO 2009). Mogensen and colleagues suggest that the development of nephropathy takes place over five stages, starting with microalbuminuria (intermediate albumin excretion rates) and hyperfunction (increased perfusion and filtration) and ending with renal failure (Mogensen, Christensen et al. 1983; DeFronzo 2003). The development of diabetic nephropathy is attributed to metabolic and hemodynamic factors, and glucose is known to be directly toxic to renal cells by activating cytokines, promoting the production of advanced glycation end products, and increasing protein kinase C activation (DeFronzo 2003). Once macroalbuminuria develops, progression to end-stage renal disease is difficult to stop, and patients must begin dialysis or obtain a kidney transplant (DeFronzo 2003). Since the kidneys also play an important role in maintaining blood pressure, diabetic nephropathy can promote the development of hypertension. Therefore, treating diabetic neuropathy requires restoring glomerular function and reducing hypertension.

Retinopathy

Diabetic retinopathy, or damage to the retina, occurs in all type 1 diabetic patients and greater than 60% of type 2 diabetic patients (Duh 2003; CDC 2007; WHO 2009). This high frequency of occurrence means diabetes is one of the leading causes of blindness in the world. Although the factors responsible for the development of retinopathy are poorly understood, early pathophysiologic changes in the retina include occlusion, dilation, and increased permeability of the retinal microvessels (Klein 2003). These changes lead to microaneurysms with little impact on vision, but progression of retinopathy leads to retinal hemorrhage and hard exudates, which do result in visual deficiency (Klein 2003). Late stages of retinopathy are characterized by severe ischemia in retinal blood vessels and an almost complete loss of vision.

Cognitive Impairment

Although it was first reported in 1922, the influence diabetes has on the central nervous system is just now beginning to be appreciated clinically and academically (Miles 1922). Diabetes impact on cognitive function ranges from mild cognitive impairment to dementia and seems to more pronounced in type 2 diabetes (Stewart and Liolitsa 1999; Brands, Kessels et al. 2006; Manschot, Brands et al. 2006; Luchsinger, Reitz et al. 2007). Type 1 and type 2 diabetic patients demonstrate deficits in performance on neuropsychological tests, and cortical atrophy was observed in patients with type 2 diabetes (Brands, Kessels et al. 2006; Manschot, Brands et al. 2006). There is such a high association between diabetes and Alzheimers disease that some researchers argue for the disease state to be called type 3 diabetes (de la Monte and Wands 2008). Few hypotheses have been proposed to explain why hyperglycemia adversely affects the central nervous system. One

plausible explanation though involves decreased neuronal insulin signaling, since insulin signaling is impaired in Alzheimers patients (Rivera, Goldin et al. 2005; Brands, Kessels et al. 2006; Manschot, Brands et al. 2006). The connection between cognitive decline and diabetes is not completely understood, but the high degree of interest in the phenomenon should help remedy that situation.

In summary, the complications associated with diabetes are generally categorized as being microvascular or macrovascular in nature. The microvascular complications include nephropathy, neuropathy, and retinopathy. Cardiovascular disease, peripheral vascular disease, and stroke comprise the macrovascular complications. The underlying pathology responsible for cognitive impairment is not completely understood and is probably a combination of microvascular and macrovascular changes. However, the diabetic complication of greatest concern for this project is neuropathy. Therefore, the focus of the remaining review is on the underlying mechanisms responsible for the development of diabetic peripheral neuropathy.

1.4 Diabetic Neuropathy

Diabetic neuropathy impacts the lives of many diabetic patients, and though estimates vary widely, nearly 70% of diabetic patients experience neuropathy at some point during the course of their disease (Pirart 1977; Dyck, Davies et al. 1999; Sinnreich, Taylor et al. 2005; CDC 2007; WHO 2009). Several types of neuropathies are associated with diabetes, about fifteen by most classification methods, and the following sections will only briefly discuss the majority of neuropathies.

1.4.a Peripheral Nerve Development and Fiber Types

In order to better understand the discussion of neuropathies, a short overview on peripheral neuron development and the peripheral nervous system (PNS) is warranted. The PNS is comprised of the somatosensory and autonomic divisions. Proper functioning of these two divisions depends on neurons and their supporting glial cells, Schwann cells. The development of the somatosensory and autonomic divisions of the peripheral nervous system requires a complex array of signals and conditions. Therefore, only the elements of peripheral neuron and glial cell development that are relevant to the development of neuropathy and the work described in this dissertation will be mentioned.

All parts of the nervous system arise from a specific region on the dorsal side of the developing embryo called the neural plate. Cells of the neural plate divide and form the neural tube, and different portions of the neural tube give rise to motor neurons and the peripheral nervous system (Martin 2003). Development of sensory neurons of the peripheral nervous system occurs from a group of cells in the dorsal neural tube known as the neural crest. Some of the cells in the neural crest are destined to become pseudounipolar neurons of the dorsal root ganglia (DRG), which transmit somatosensory information, and Schwann cells. A group of growth factors known as the neurotrophins are critical in determining whether a neural crest cell will become a sensory neuron. Neurotrophin-3 (NT-3) provides a mitogenic effect for migrating neural crest cells destined to become sensory neurons (Kalcheim, Carmeli et al. 1992; Pinco, Carmeli et al. 1993). Brain derived neurotrophic factor (BDNF) has also been shown to promote survival and neuronal differentiation of neural crest cells (Kalcheim and Gendreau 1988). Many of the migrating neural crest cells respond to NT-3 and BDNF will become DRG neurons that require nerve growth factor (NGF) for survival (Johnson, Gorin et al. 1980). Aberrations in these signaling pathways

during development results in malformation of the peripheral nervous system and, as will be made evident, irregularities after development can have devastating effects on the PNS. It is important to note that NT-3, BDNF, and NGF are not the only factors critical for the development of sensory neurons. However, these growth factors also play critical roles in maintaining proper function of the peripheral nervous system.

A key component to the development of the PNS is the development of Schwann cells. Schwann cells function in the production of the myelin sheath that insulates axons and allows for rapid saltatory conduction. Like sensory neurons, Schwann cells differentiate from cells of the neural crest. However, the differentiation and survival of Schwann cells depends on different growth factors and signaling molecules. Schwann cell differentiation and development requires an extensive network of transcription factors and signaling molecules, including Sox-10, Oct-6, Krox-20, insulin-like growth factor, and platelet derived growth factor (Jessen and Mirsky 1998). Jessen and Mirsky propose three stages for the development of mature Schwann cells (Jessen and Mirsky 1997). First is the formation of Schwann cell precursors from neural crest cells. Second is the development of immature Schwann cells from precursors, and the final stage is the transition from immature Schwann cell to mature, non-myelinating or myelinating Schwann cell. During each stage, neuregulin-1 and the ErbB2/ErbB3 receptor pair mediate the initiation of key signaling pathways that promote the progressive differentiation of neural crest cells into mature, myelinating Schwann cells (Shah, Marchionni et al. 1994; Dong, Brennan et al. 1995; Dong, Sinanan et al. 1999). Once mature Schwann cells have developed, those Schwann cells associated with an axon of at least one micron in diameter begin the process of myelination. The molecular mechanisms guiding this process of axon/Schwann cell pairing and myelin formation are still

being worked out. However, the extent of myelin formation depends on NRG-1 signaling (Woldeyesus, Britsch et al. 1999; Michailov, Sereda et al. 2004). That is, myelin thickness is directly proportional to NRG-1 expression. The current thinking is that the contact between a mature Schwann cell and a sensory axon initiates bi-directional signaling pathways which enhance the transcription of axonal signals and the myelin proteins P₀, myelin basic protein, and myelin-associated glycoprotein (Colello and Pott 1997). Adequate transcription and translation of these proteins and continued activation of signaling pathways in the axon and Schwann cell then leads to compaction of the myelin sheath.

Once terminal differentiation of neurons and Schwann cells occurs, the myelination program can be initiated, and functional nerves that are capable of transmitting sensory information will then be present. Not all neurons are the same size or have the same parameters though. According to Joseph Erlanger and Herbert Gasser, the afferent fibers of the peripheral nervous system are organized into four classes based on size and conduction velocity (Gasser 1941). The four afferent classes include A- α , A- β , A- δ , and C-fibers. A- α fibers are the largest fiber type in the PNS, they range from 13-20 μm in diameter, and predominantly transmit position sense information from mechanoreceptors (Martin 2003). These fibers demonstrate the greatest degree of myelination, and therefore transmit action potentials at higher velocities than the other fiber types, from 70 m/s to 120 m/s (Møller 2006). A- β fibers are slightly smaller than A- α fibers, ranging from 6-12 μm in diameter (Martin 2003). A- β fibers are heavily myelinated, though to a lesser degree than A- α fibers, and mostly transmit touch and position sense information at velocities between 30-70 m/s (Martin 2003; Møller 2006). A- δ fibers are smaller yet, ranging from 1 μm to 5 μm , and are thinly myelinated or unmyelinated. These fibers function in the transmission of temperature

sense and pain. Action potential propagation in A- δ fibers occurs at velocities ranging from 12-30 m/s (Møller 2006). The smallest fiber type of the peripheral nerve fibers is the C-fiber, 0.2-1.5 μ m (Martin 2003). C-fibers are not myelinated and generally transmit temperature, pain, and itch sensations. Such information travels at slower velocities close to 0.5 m/s to 2 m/s (Møller 2006). The differences in these fiber types and their sensory modalities are an important factor in the manifestation of neuropathy associated with diabetes.

1.4.b Classification of Diabetic Neuropathy

Currently there are two prominent classification schemes for diabetic neuropathy. One scheme focuses on the clinical manifestation of the neuropathy while the other focuses on the underlying pathophysiology responsible for the neuropathy (Sinnreich, Taylor et al. 2005; Tracy and Dyck 2008). The main distinguishing factor in the clinical classification scheme is the symmetry of the neuropathy. A portion of diabetic neuropathies manifest on both sides of the body in nearly equal proportion and are thus considered symmetrical. Not surprisingly, certain diabetic neuropathies only manifest on one side of the body. These neuropathies are therefore considered asymmetrical. The scheme based on the underlying pathophysiology is a little more complicated. In this scheme, neuropathies fall into one of the following categories: metabolic-microvascular-hypoxic, inflammatory immune, compression and repetitive injury, complications of diabetes, and treatment related neuropathies. Each classification scheme has its advantages and disadvantages, which are not relevant to the current discussion. However, the neuropathies found in both schemes are relevant and will be discussed in further detail below.

Diabetic Sensorimotor Polyneuropathy

Diabetic sensorimotor polyneuropathy, sometimes referred to as diabetic distal symmetrical polyneuropathy or diabetic polyneuropathy and usually abbreviated DPN, is the most common form of diabetic peripheral neuropathy. The prevalence of DPN is approximately 55% and 45% for type 1 and type 2 diabetes, respectively, and patients present with either positive or negative symptoms (Dyck, Kratz et al. 1993). Positive symptoms are painful and include a burning sensation, pins and needles sensations, and hypersensitivity to touch. Negative symptoms on the other hand are painless and include thermal hypoalgesia, loss of vibration or pain sense, and numbness.

A sizeable portion of diabetic patients with sensorimotor polyneuropathy are initially asymptomatic. In the Rochester Diabetic Neuropathy Study, Dyck *et al.* report that 15% of the patients with clinical signs of polyneuropathy did not present with symptoms of the condition (Dyck, Kratz et al. 1993). This phenomenon suggests that hyperglycemia induced nerve damage occurs before it is noticed by an individual, that duration of diabetes influences development of polyneuropathy and that symptomatic polyneuropathy may be preventable. Duration of diabetes influences the development of complications and the Diabetes Control and Complications Trial (DCCT) concluded that intensive diabetes treatment significantly delays or prevents the development of polyneuropathy (1988; Ziegler, Mayer et al. 1988; 1995; 1995; Valensi, Giroux et al. 1997; Pambianco, Costacou et al. 2006). Being the most common neuropathy associated with diabetes, sensorimotor polyneuropathy receives the most attention in academic and clinical discussions of diabetic neuropathies.

As mentioned earlier, diabetic sensorimotor polyneuropathy manifests mainly as a distal symmetric condition. That is, distal nerves, or those farthest from the CNS, on both sides of the body will be damaged before the more proximal nerves close to the midline.

This manifestation leads to what is classically referred to as the glove and stocking distribution of nerve damage, wherein the feet and hands experience nerve damage first (Tracy and Dyck 2008). Progression of the neuropathy leads to proximal spreading of the nerve damage up the extremities. Because of the multifactorial nature of the etiology of diabetic polyneuropathy, no single test has been found to adequately identify nerve damage. Therefore, the most common tools used to identify and diagnose polyneuropathy include testing electrophysiologic function and quantitative sensory thresholds (QSTs) (1992; 1992; 1993; 1995; 1995; 1995). Electrophysiologic measures are also referred to as nerve conduction velocity tests and generally include parameters of compound muscles action potentials (CMAPs) and sensory nerve action potentials (SNAPs). Diabetic patients subjected to nerve conduction velocity tests demonstrate significant decreases in both motor and sensory conduction velocities. Motor nerve conduction in the median and tibial nerves of diabetic patients are 15-20% less than in non-diabetic patients (Kimura, Yamada et al. 1979). Such deficits are also observed for sensory nerve conduction velocity. Furthermore, it is not abnormal for the motor nerve conduction velocity in patients with longstanding neuropathy to be around 30 m/s, where it would normally be closer to 55-60 m/s (Gilliat and Willison 1962; Arezzo and Zotova 2002). The set of tools used to diagnose polyneuropathy are QST tests, which include vibratory perception, touch pressure, and heat and cold discrimination. As may be expected, diabetic patients also demonstrate deficits in these measures. Nerve dysfunction is exhibited to a greater degree when measuring warm and cold perception and temperature discrimination (Bertelsmann, Heimans et al. 1985; Ziegler, Mayer et al. 1988). Between 30 and 60% of diabetic patients demonstrate deficits in one or more QST assessments (Navarro and Kennedy 1991). Occasionally, pathological techniques

such as nerve biopsy and morphometric analysis of epidermal innervation (ENFD) are employed to assess nerve damage. It is important to note that although ENFD techniques have several disadvantages, their use has increased recently. A consequence of this increased use has been a reduction in cost and an increase in reproducibility. Therefore such techniques are becoming standard clinical criteria for the diagnosis of DPN. Although the tools used to assess diabetic polyneuropathy are constantly evolving, the most agreed upon methods to date are those mentioned.

Since diabetes encompasses several different disorders with distinctive etiologies, it should not be surprising that the development of polyneuropathy is also multifactorial. The etiology of polyneuropathy primarily involves metabolic and microvascular changes, but identifying any one mechanism has proved to be difficult. The leading hypotheses explaining the development of polyneuropathy include an increase in flux through the polyol and hexosamine pathways, increased production of advanced glycation end products (AGEs), increased activation of protein kinase C (PKC) and altered neurotrophism. All of these processes likely play a role in the development of neuropathy, but how they relate to each other and their specific contribution to the development of clinical neuropathy is not completely understood. Some details regarding these hypotheses will be given later. The important thing to understand at this point is that the cellular mechanisms responsible for the development of diabetic sensorimotor polyneuropathy have not been fully elucidated.

Diabetic Autonomic Neuropathy

Diabetic autonomic neuropathy (DAN), as the name implies, primarily affects the autonomic nervous system (ANS), and because of this can have devastating effects on all of the major organ systems of the body. Although symptoms of autonomic neuropathy are more

common in type 1 diabetic patients, autonomic impairment is more prevalent in type 2 diabetics. Approximately 73% of type 2 diabetics and 54% of type 1 diabetic patients suffer from DAN (Low, Benrud-Larson et al. 2004). Severe autonomic dysfunction occurs in significantly lower proportions though, affecting about 15% of diabetic patients (Low, Benrud-Larson et al. 2004). The range of organ dysfunctions associated with DAN is vast and unfortunately the mechanisms responsible for DAN are not completely understood.

The clinical manifestations of DAN are quite variable and impact numerous organ systems. The organ systems most commonly affected by autonomic neuropathy include the pupil, sweat glands, genitourinary system, gastrointestinal (GI) tract, adrenal gland, and the cardiovascular system (Vinik 2003). A more extensive list of clinical manifestations can be seen in Table 1.2 (Vinik 2003). Cardiovascular specific dysfunctions associated with DAN are commonly referred to as cardiovascular autonomic neuropathy (CAN) and include abnormalities in heart rate control and neurovascular dynamics (Vinik 2003; Vinik and Mehrabyan 2003). Since the GI tract spans a large portion of the body and is innervated at every level by the autonomic nervous system, the presentation of GI tract dysfunction is quite variable. GI dysfunction may include dysphagia, abdominal pain, nausea, vomiting, malabsorption, fecal incontinence, diarrhea, and constipation (Vinik 2003; Vinik and Mehrabyan 2003). Diabetes is also the most common cause of erectile dysfunction (ED), which serves as an indicator of systemic vascular health. Estimates for the proportion of diabetic men with ED range from 35% to 75% (McCulloch, Campbell et al. 1980). Other serious autonomic dysfunctions that can be associated with DAN include neurogenic bladder, abnormalities in hypoglycemia responsiveness, and tissue perfusion (Vinik 2003; Vinik and Mehrabyan 2003). The organs and tissues controlled by the autonomic nervous system are

numerous, and diabetic autonomic neuropathy can have significant influence on quality of life.

Table 1.2: Various clinical manifestations of diabetic autonomic neuropathy.

Clinical Manifestation
Cardiovascular
Tachycardia, exercise intolerance
Cardiac denervation
Orthostatic hypotension
Gastrointestinal
Esophageal dysfunction
Gastroparesis diabeticorum
Diarrhea
Constipation
Fecal incontinence
Genitourinary
Erectile dysfunction
Retrograde ejaculation
Cystopathy
Neurogenic bladder
Neurovascular
Heat intolerance
Gustatory sweating
Dry skin
Impaired skin blood flow
Metabolic
Hypoglycemia unawareness
Hypoglycemia unresponsiveness
Hypoglycemia associated autonomic failure
Papillary
Decreased diameter of dark adapted pupil
Argyll-Robertson type pupil

Why autonomic nerves are targeted in some cases of diabetes and not others is not known, and the mechanisms responsible for the development and progression of diabetic autonomic neuropathy are poorly understood. Persistent hyperglycemia is the initiating factor that leads to metabolic and microvascular changes, which are thought to be responsible for DAN. Similar to sensorimotor polyneuropathy, the pathogenesis of DAN is attributed to

direct nerve damage caused by increased flux through the polyol and hexosamine pathways, increased production of AGEs, increased activation of PKC, and altered neurotrophism (Vinik 2003; Vinik and Mehrabyan 2003). Again, how these metabolic and microvascular changes are thought to induce nerve damage will be discussed in greater detail below.

1.4.c Mechanisms of Diabetic Neuropathy Development

Though the etiological development of DPN is not completely understood, there is agreement that the pathogenesis of the DPN is multifactorial in nature. The discussion to follow will focus on the proposed mechanisms responsible for the development of DPN, particularly diabetic sensorimotor polyneuropathy, but these mechanisms likely apply to DAN as well.

Polyol Pathway

The polyol pathway utilizes aldose reductase to reduce glucose and toxic aldehydes, produced from reactive oxygen species (ROS), into sorbitol and inactive alcohols respectively. Sorbitol produced by the polyol pathway is then converted to fructose by sorbitol dehydrogenase (see Figure 1.1). In order for the reduction of glucose by aldose reductase to occur, nicotinamide adenine dinucleotide phosphate (NADPH) must be present. Under normal conditions the polyol pathway metabolizes only a small portion of glucose because aldose reductase has a low affinity for glucose. During periods of hyperglycemia, however, excess availability of glucose leads to significantly increased flux through the polyol pathway. This increased flux is thought to be detrimental to microvascular cells that supply nerves and their supporting glia in several ways. One early hypothesis was that excess sorbitol induces osmotic damage in microvascular cells. It has been shown however, that sorbitol concentrations in diabetic microvessels and nerves are not sufficient to cause

osmotic damage (Brownlee 2003). A second hypothesis claimed that increased flux through the polyol pathway reduced Na^+/K^+ ATPase activity. Again, this has been shown not to be the case. Hyperglycemia induced activation of PKC is responsible for reduced Na^+/K^+ ATPase activity, which will be discussed in more detail below (Xia, Kramer et al. 1995). It has also been proposed that oxidation of sorbitol by NAD^+ leads to higher concentrations of triose phosphates,

which lead to increased formation of methylglyoxal, a precursor for the formation of advanced glycation end products (AGEs) (Brownlee 2003). Formation of AGEs and their role in neuropathy will also be discussed below.

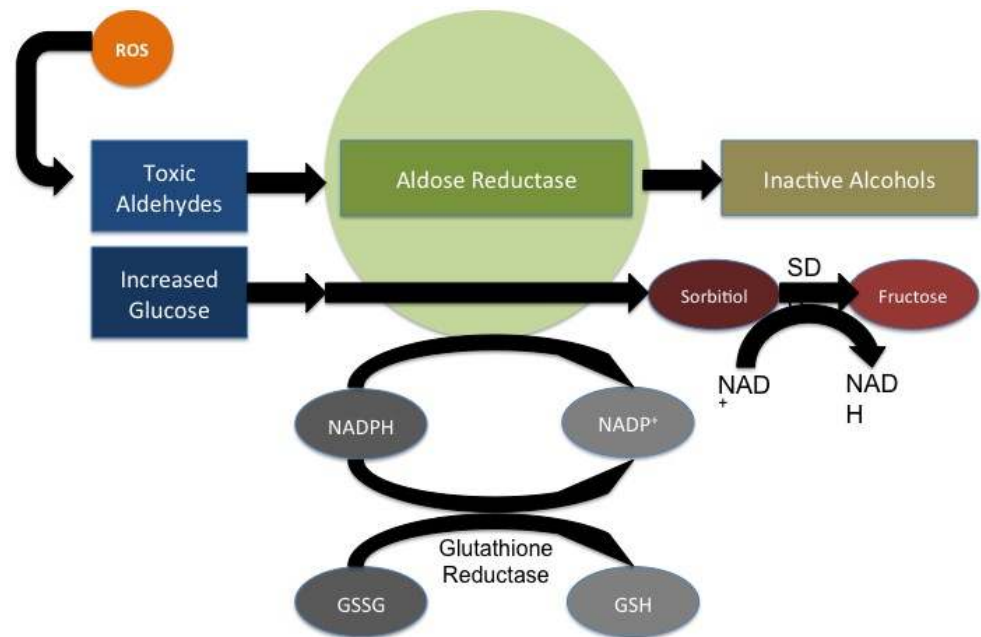


Figure 1.1: Polyol Pathway. Under hyperglycemic conditions, aldose reductase converts glucose into sorbitol, which is then broken down into fructose. This reaction requires NADPH. Therefore, during periods of hyperglycemia, NADPH is consumed and ROS accumulate as a result. (Brownlee 2001)

The last hypothesis concerned with how increased flux through the polyol pathway is detrimental to microvessels involves the reaction cofactor NADPH. As already noted, aldose reductase requires NADPH for the reduction of glucose. Increased flux through the polyol pathway consumes available NADPH. Since NADPH is required for the regeneration of

glutathione, decreased availability of NADPH promotes an increase in oxidative stress by reducing glutathione levels (Brownlee 2003). Therefore, persistent hyperglycemia leads to increased metabolism of glucose via the polyol pathway and induces nerve damage by promoting the formation of AGEs and impairing the elimination of ROS.

Hexosamine Pathway

Like the polyol pathway, the hexosamine pathway processes little glucose under normal conditions. However, excess glucose is shunted through the hexosamine pathway during periods of hyperglycemia. Shunting to the hexosamine pathway occurs after glucose has been converted to fructose-6-phosphate during glycolysis (see Figure 1.2). Instead of continuing on with glycolysis, fructose-6-phosphate is redirected to the hexosamine pathway, where it is converted to glucosamine-6-phosphate and then UDP-N-acetylglucosamine (Voet, Voet et al. 2006).

UDP-N-acetylglucosamine is then used for the formation of *O*-linked glycoproteins, which modify transcription factors, nuclear proteins, and cytoplasmic

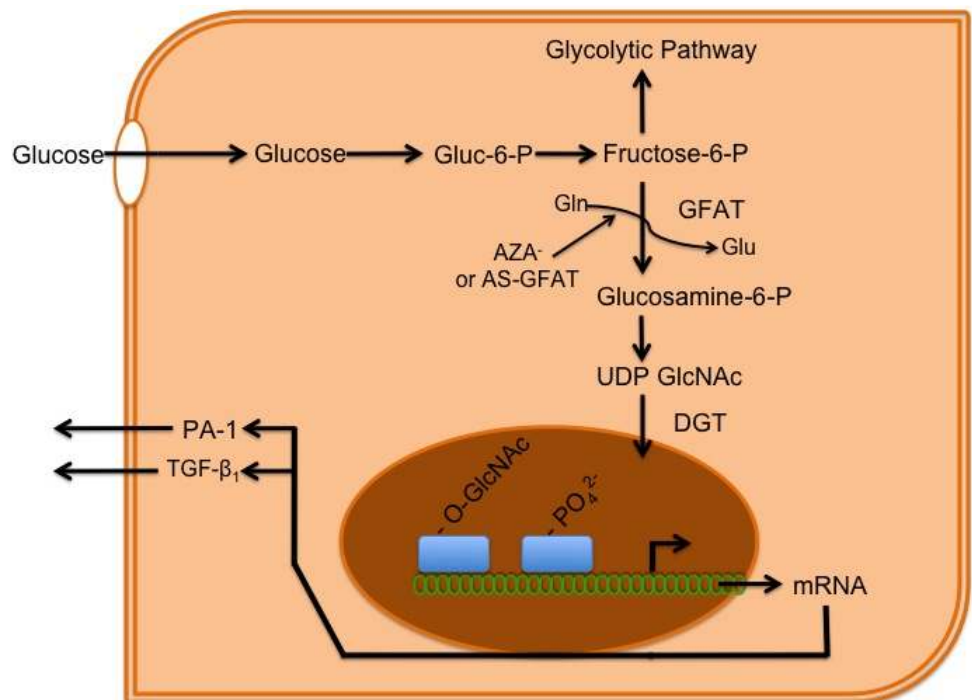


Figure 1.2: Hexosamine Pathway. Under hyperglycemia conditions, excess fructose-6-phosphate from glycolysis gets shuttled to the hexosamine pathway where it is used in the formation of glycoproteins. (Brownlee 2001)

proteins (Brownlee 2003). Exactly how increased flux through the hexosamine pathway elicits nerve damage is not known, but the current thinking is that several transcription factors regulating genes involved in the development of diabetic neuropathy are modified by O-linked glycoproteins in a way that promotes the development of neuropathy.

Protein Kinase C Activation

Diacylglycerol (DAG) is a second messenger in microvascular cells functions in the activation of nine of the eleven members of the PKC family (Brownlee 2003). In the fifth step of glycolysis, dihydroxyacetone phosphate is converted to glyceraldehyde-3-phosphate, which is converted to 1,3-bisphosphoglycerate in the sixth step (Voet, Voet et al. 2006). When the intracellular concentration of glucose increases significantly, as is the case during diabetes, more glyceraldehyde-3-phosphate is available in the cell and some of it is used to synthesize DAG. Therefore, hyperglycemia increases the de novo synthesis of DAG leading to increased PKC activation (Brownlee 2003). Increased PKC activation has several detrimental effects on the cell. As noted above, PKC activation leads to reduced Na^+/K^+ ATPase activity. PKC activation increases phospholipase A_2 activity and the production of the Na^+/K^+ ATPase inhibitors, arachidonate and PGE_2 (Xia, Kramer et al. 1995). The Na^+/K^+ ATPase plays a critical role in maintaining membrane potential and impaired Na^+/K^+ ATPase activity alters neuronal membrane dynamics and cell integrity. Increased activation of PKC can also lead to blood flow abnormalities, vascular problems, inflammation, and the generation of ROS (Brownlee 2003). As described in Figure 1.3, PKC activation limits nitric oxide (NO) production and decreases blood flow and increases blood pressure. PKC induced increases in vascular endothelial growth factor (VEGF), transforming growth factor β (TGF- β), and plasminogen activator inhibitor 1 (PAI-1) induce changes in vascular permeability,

capillary occlusion, and vascular occlusion, respectively. Increased expression of pro-inflammatory genes also occurs as a result of increased PKC activation. Lastly, increased PKC activation results in increased oxidation of NADH and NADPH, which promotes the generation of reactive oxygen species. However, given the role of PKC in numerous biochemical pathways, PKC inhibitors have been problematic for treating DPN.

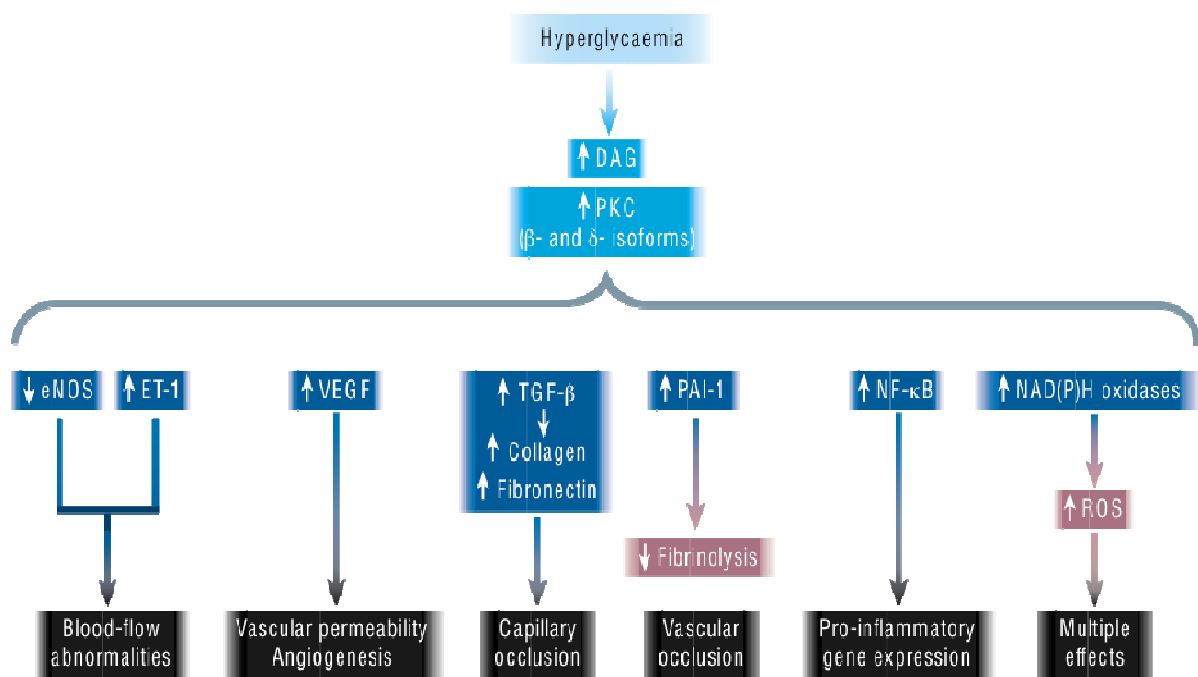


Figure 1.3: Consequences of Increased Protein Kinase C Activation. Hyperglycemia results in an increase in DAG, which activates PKC. Increased PKC activation leads to blood flow and vascular abnormalities, inflammation, and the generation of ROS.

Non-enzymatic Formation of Advanced Glycation End Products

Glycation, or the addition of a monosaccharide or carbonylic compound to an organic molecule, is a normal metabolic process. Glycation sets off a chain of non-enzymatic reactions that result in the formation of AGEs. Accumulation of AGEs coincides with aging but can also be harmful to cells if occurring in excess (Nass, Bartling et al. 2007).

Hyperglycemia has been shown to initiate the accumulation of intracellular and extracellular AGEs and is implicated in the development of several diabetic complications, especially neuropathy (Brownlee 2003). Indeed, AGE accumulation has been shown to be detrimental to both neurons and Schwann cells (Vlassara, Brownlee et al. 1983; Vlassara, Brownlee et al. 1984; Takeuchi, Bucala et al. 2000; Misur, Zarkovic et al. 2004; Sekido, Suzuki et al. 2004). During glycolysis, glucose can be metabolized into reactive dicarbonyls including 3-deoxyglucosone (3-DG), N ϵ -(carboxymethyl) lysine (CML), N ϵ -(carboxyethyl) lysine (CEL), and methylglyoxal (MG) (Thornalley 2002; Brownlee 2003). The reactive dicarbonyl glyoxal arises from the autoxidation of glucose and fructose is further metabolized to 3-DG (Thornalley 2002; Brownlee 2003). These reactive dicarbonyls go on to form AGEs. Once AGEs have formed, they are thought to carry out their damaging affects in several ways including altering protein function, interfering with interactions between the cell and extracellular matrix, and modifying gene expression.

AGEs can form on proteins in the extracellular matrix including collagen type IV and laminins (Brownlee 2003). Formation of AGEs on these proteins stiffens the extracellular matrix and compromises vessel function (Monnier, Kohn et al. 1984). Furthermore, formation of AGEs in vascular and neuronal tissues interferes with molecular interactions in the extracellular matrix and between the cell and the extracellular matrix (Brownlee 2003). Interference of this sort can result in reduced cell adhesion and reduced neurite outgrowth (Haitoglou, Tsilibary et al. 1992; Federoff, Lawrence et al. 1993). The full extent of damage caused by modifying proteins in the extracellular matrix and reducing interactions in the extracellular matrix are not completely understood, but can be detrimental to microvessels and neurons.

The receptor for advanced glycation end products (RAGE) and the ability of AGEs to interact with numerous cellular receptors allow AGEs to mediate further, long-term cell damage. The most prominent transcription factors affected by RAGE activation are NF κ B and AP-1, which promote the expression of proinflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) (Toth, Martinez et al. 2007). Increased expression of IL-6 and TNF- α is thought to mediate AGE associated tissue damage. Evidence from RAGE knockout mice (RAGE^{-/-}) supports this hypothesis. Increasing duration of diabetes correlates with increased AGE accumulation, the progression of peripheral nerve dysfunction, and demyelination (Toth, Rong et al. 2008). There is no such relationship in RAGE^{-/-} mice though. Diabetic RAGE^{-/-} mice demonstrate significantly less AGE accumulation and increased compound muscle action potential amplitude, motor nerve conduction velocity, sensory nerve action potential amplitude, and sensory nerve conduction velocity compared to their wild-type counterparts (Toth, Rong et al. 2008). Furthermore, diabetic RAGE^{-/-} mice do not demonstrate the demyelination observed in diabetic wild-type mice (Toth, Rong et al. 2008).

Convergence of Pathogenetic Mechanisms on ROS Production

The increase in flux through the polyol and hexosamine pathways, increased activation of PKC, and increased formation of AGEs under hyperglycemic conditions seems to be a compensatory mechanism of cells. Under euglycemic conditions the majority of glucose is metabolized through the glycolytic pathway. During hyperglycemia though, cells compensate for the increased concentrations of glucose and its metabolites by utilizing other ways to metabolize these molecules. Unfortunately, this increase in glucose metabolism and extraordinary metabolism has detrimental effects on nerves. Damage to the neurons is

mediated primarily by ROS. In the context of hyperglycemia, ROS are generated in several ways (see Figure 1.4). First, increased glycolysis translates into increased activity of the tricarboxylic acid cycle (TCA cycle) and therefore oxidative phosphorylation, which produces superoxide in the electron transport chain. This initial production of ROS inhibits the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which prevents the completion of glycolysis, and accumulation of several glycolytic metabolites, including DAG. Increases in DAG concentration result in increased PKC activation, which alters protein function and promotes the generation of ROS. Third, increased activity of aldose reductase uses up available glutathione, which reduces ROS. Therefore, by preventing the reduction of ROS, increased flux through the polyol pathway increases the generation of ROS. Finally, increases in flux through the hexosamine pathway leads to the modification of transcription factors and cytosolic proteins that influence the generation of ROS. The accumulation of ROS, then, continues to alter the function of proteins and transcription factors that modify neurotrophism and gene expression. Whether or not hyperglycemia alters gene expression and neurotrophism in other ways is not known. Regardless, the role for neurotrophism in the development of diabetic peripheral neuropathy is a relatively recent development, and the forthcoming evidence in support of this view may prove to be one of the missing pieces in our search for the pathogenic mechanisms responsible for the development of DPN.

Altered Neurotrophism

During the growth and development of the peripheral nervous system, growth factors and their receptors serve important functions in regulating certain aspects of the developmental process. By helping maintain neurons and Schwann cells, growth factors serve critical functions after the developmental process has terminated. Endogenous growth factors promote survival of neurons and regeneration after injury. Only recently has it been proposed that hyperglycemia could elicit changes in the expression or availability of such growth factors and thus, have ramifications for peripheral nerve function. Indeed, the neurotrophins, cytokine like growth factors, insulin like growth factors, vascular endothelial growth factor, and their respective receptors have all been implicated in the pathogenesis of diabetic neuropathy (Leininger, Vincent et al. 2004). Expression or activity of nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-4/5 (NT-4/5), insulin like growth factor-I, and ciliary neurotrophic factor (CNTF) is significantly reduced as a result of streptozotocin (STZ) induced hyperglycemia in mouse models of diabetes (Hellweg and Hartung 1990; Mizisin, Bache et al. 1997). Conversely, neurotrophin-3 (NT-3) and VEGF demonstrate increased expression (Calcutt, Muir et al. 1992; Foster, Robertson et al. 1994; Wuarin, Guertin et al. 1994; Rodriguez-Pena, Botana et al. 1995; Samii, Unger et al. 1999; Ha, Kim et al. 2001). The expression of receptors for several of the growth factors, such as TrK A, TrK B, TrK C, and p75 also undergo changes as a result of experimental diabetes (Leininger, Vincent et al. 2004). Moreover, most of these changes correspond with nerve dysfunction or adverse morphological changes. Based on these data several clinical trials employing growth factors as a treatment for DPN were undertaken. Early phase I clinical trials using recombinant human nerve growth factor (rhNGF) showed some efficacy

in alleviating symptoms of diabetic peripheral neuropathy (Apfel, Kessler et al. 1998). However, more tightly controlled phase III trials using rhNGF though, did not improve the signs or symptoms of diabetic peripheral neuropathy (Apfel, Schwartz et al. 2000). Phase I-II clinical trials using BDNF also showed that this growth factor was ineffective in treating diabetic peripheral neuropathy (Wellmer, Misra et al. 2001). A study exploiting NT-3 was also undertaken, but never published, suggesting results were less than favorable (Leininger, Vincent et al. 2004). From these studies it is evident that changes in the expression or activity of growth factors or their corresponding receptors can alter the neuronal environment and result in dysfunction, but evidence from clinical trials suggests that other factors are involved as well.

The lack of efficacy in clinical trials employing neuronal growth factors highlights the need for further research in this area. However, it is important to note that neurons and overall nerve function also depend on Schwann cells. How hyperglycemia influences growth factor signaling in Schwann cells has yet to be realized. The primary growth factors for Schwann cells are the neuregulins, which bind to the ErbB family of receptors. Dobrowsky *et al.* proposed that hyperglycemia could alter neuregulin signaling via changes in the expression or activity of these growth factors and their corresponding receptors, and that this may contribute to the development of neuropathy (Dobrowsky, Rouen et al. 2005). This hypothesis will be expanded upon in detail below.

1.5 Altered Neuregulin Signaling in DPN

Almost all studies relating to altered neurotrophism have focused on neuronal growth factor signaling. Consequently there is a significant gap in our understanding of how diabetes and hyperglycemia influence growth factor signaling in Schwann cells. The primary

growth factors for Schwann cells are the neuregulins, which mediate their effects by binding to the ErbB family of receptors and initiating various signaling cascades.

There are four members of neuregulin family of growth factors, neuregulin-1 (NRG-1), neuregulin-2 (NRG-2), neuregulin-3 (NRG-3), and neuregulin-4 (NRG-4). Neuregulin-1 is the most studied of the group and has at least fifteen variants due to alternative splicing and differential promoter usage (Lemke 1996). These fifteen variants have been grouped into three types of NRG-1, all of which contain an EGF like domain. Neuregulin-1 type I (NRG-1 type I) has a glycosylation-rich segment and is also known as acetylcholine receptor inducing activity (ARIA), neu differentiation factor (NDF), and heregulin (Adlkofer and Lai 2000). Neuregulin-1 type II (NRG-1 type II) is also referred to as glial growth factor (GGF), and neuregulin-1 type III (NRG-1 type III) contains a cysteine rich domain and is sometimes called sensory motor derived factor (SMDF) (Adlkofer and Lai 2000). The NRG-1s are generally bound to axonal membrane, but can be proteolytically cleaved and released into the extracellular fluid. Interestingly, the proteolytic cleavage of the NRG-1s is enhanced by PKC activation (Loeb, Susanto et al. 1998). Whether or not cleavage of the NRG-1s is related to the pathophysiologic development of DPN is not known, but could be one of the ways in which hyperglycemia induced PKC activation modifies neurotrophic factors.

Cleaved and transmembrane NRG-1s both bind to the ErbB family of receptors.

The ErbB receptors are members of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. There are four members of this family: ErbB1, which is also known as the EGFR, ErbB2, ErbB3, and ErbB4*. The expression of ErbB1 and ErbB4 is negligibly low in adult Schwann cells and these two receptors are thought to contribute

* The ErbB receptors are sometimes referred to as HER1, HER2, HER3, and HER4, where HER stands for human epidermal growth factor receptor. The HER designation will herein be dropped and the receptors will only be referred to as the ErbBs.

very little to neuregulin induced signaling in these cells (Grinspan, Marchionni et al. 1996; Syroid, Maycox et al. 1996). ErbB2 is a fully functional receptor with the exception that it has no ligand-binding domain (Sliwkowski, Schaefer et al. 1994; Alroy and Yarden 1997; Klapper, Glathe et al. 1999). ErbB3 on the other hand, has a ligand-binding domain, but lacks functional kinase activity (Guy, Platko et al. 1994). Since ErbB1 and ErbB4 expression is low in adult Schwann cells, the only relevant receptors for the current discussion are the ErbB2 and ErbB3 receptors.

NRG-1 is bound to the axonal membrane and the ErbB receptors are transmembrane receptors expressed in Schwann cells. Transduction of the NRG-1 signal occurs when it binds to ErbB3. Ligand binding induces receptor dimerization, cross-phosphorylation, and the recruitment and activation of various signaling molecules. Members of the ErbB family of receptors can form homodimers or heterodimers with other members of the ErbB family, but ErbB2 and ErbB3 must form heterodimers to form a functional receptor unit in Schwann cells. Generally, ligand binding induces a conformational change in the receptor that promotes dimerization. Although ErbB2 lacks a ligand-binding domain, it is able to form heterodimers because its dimerization domain is always extended (Cho, Mason et al. 2003; Garrett, McKern et al. 2003). It is as if ErbB2 is constitutively bound to a ligand. Then, when NRG-1 binds ErbB3, a conformational change takes place such that the dimerization domain of ErbB3 is extended. Interaction of the dimerization domains of both receptors leads to heterodimerization. Once heterodimerization has taken place, tyrosine residues in the intracellular domain of the receptors become phosphorylated. Signaling complexes are assembled at these sites and phosphorylation of these residues can lead to the activation of numerous signaling pathways including the phosphatidylinositol-3-phosphate kinase (PI3K)

pathway and the mitogen activated protein kinase (MAPK) pathway (Pinkas-Kramarski, Shelly et al. 1998; Cho, Mason et al. 2003; Monje, Athauda et al. 2008). The various signaling networks initiated by ErbB2 activation allow it to be involved in many developmental and pathological processes.

One of the developmental processes where ErbB2 and neuregulin signaling play an important role is the development of the peripheral nervous system and myelination. Mutant mice lacking ErbB2 demonstrate an almost complete loss of Schwann cells, and axons from mice completely lacking NRG-1 type III do not undergo myelination (Woldeyesus, Britsch et al. 1999). These are extreme examples though and highlight the need for neuregulin/ErbB2 signaling in the development of the PNS. More subtle examples showing how important neuregulin/ErbB2 signaling is for the development of the PNS involve the extent of myelination. Mice heterozygous for NRG-1 type III (NRG-1 type III^{+/-}) have significantly more unmyelinated axons than wild-type mice (Taveggia, Zanazzi et al. 2005). Furthermore, the axons that are myelinated in NRG-1 type III^{+/-} mice have considerably less myelin than wild-type mice (Michailov, Sereda et al. 2004; Taveggia, Zanazzi et al. 2005). Transgenic mice that over-express NRG-1 type III have significantly thicker myelin sheaths than wild-type mice (Michailov, Sereda et al. 2004). Neuregulin-1 levels are not the only determinant of myelin thickness though. ErbB2 levels also contribute to the ensheathment fate of axons. Since ErbB2 has functional kinase activity it is normally the dominant receptor in Schwann cells. Introducing ErbB4 to Schwann cells though, modifies this pattern. Expression of a dominant-negative ErbB4 (dn-ErbB4), in which the tyrosine kinase activity has been removed, in Schwann cells blocks ErbB2/ErbB3 signaling (Chen, Rio et al. 2003). Blocking ErbB2 signaling in this way results in greater numbers of small axons and significantly

reduced myelin thickness (Chen, Rio et al. 2003). Inducing the over-activation of ErbB2 has similar effects on myelination. Infection with the bacterium *M. leprae* results in enhanced ErbB2 activation (Tapinos, Ohnishi et al. 2006). This increased ErbB2 activity corresponds with decreased myelin thickness and increased demyelination (Tapinos, Ohnishi et al. 2006). From these studies it is evident that any modification that results in abnormal neuregulin-1/ErbB2 signaling causes significant changes in myelin thickness. Too little NRG-1 or ErbB2 and axons do not myelinate adequately. Too much NRG-1 and ErbB2 and axons become hypomyelinated. It seems that there is a specific range of NRG-1/ErbB2 signaling that must be maintained in order for Schwann cells to myelinate axons at the appropriate level.

The results from studies manipulating NRG-1/ErbB2 signaling suggest that molecules affecting this signaling could have adverse effects on myelination and nerve function. Tan and colleagues have shown that hyperglycemia influences neuregulin-1 signaling (Tan, Rouen et al. 2003). Primary Schwann cells grown in hyperglycemic conditions and stimulated with NRG-1 are more mitogenically active than cells not stimulated with NRG-1 or cells not grown in hyperglycemic conditions (Tan, Rouen et al. 2003). Furthermore, ErbB2 activation is significantly increased in primary Schwann cells grown in excessive concentrations of glucose and stimulated with NRG-1 (Tan, Rouen et al. 2003). As we now know, a significant alteration in ErbB2 signaling has a significant impact on myelination, and these results suggest that hyperglycemia directly or indirectly impacts NRG-1/ErbB2 signaling.

One means by which hyperglycemia could yield its effect on NRG-1/ErbB2 signaling is through the protein Caveolin-1. Caveolin-1 is the primary protein of caveolae,

small, cave-like invaginations in the plasma membranes and serves many roles in many cell types. The main functions of Caveolin-1 include vesicular endocytosis and transcytosis, cholesterol homeostasis, signal transduction, and protein regulation (Cohen, Hnasko et al. 2004). One of the most important of these functions is signal transduction. Caveolin-1 is able to bind numerous intracellular signal transduction molecules, including the Src family of kinases, protein kinase A (PKA), PKC, MAPK, PI3K, AKT, G proteins, Ras, and adenylyl cyclase among others (Patel, Murray et al. 2008). Caveolin-1 has also been shown to interact with numerous receptors, including various G-protein coupled receptors (GPCRs), EGFR, ErbB2, and the insulin receptor (Li, Okamoto et al. 1995; Couet, Sargiacomo et al. 1997; Engelman, Lee et al. 1998; Gustavsson, Parpal et al. 1999). Several of these signaling molecules and receptors play important roles in neuregulin-1/ErbB2 signaling, which suggests that a loss of Caveolin-1 could alter this signaling. Indeed, this is what is observed under experimental conditions. Forced down-regulation of Caveolin-1 in primary Schwann cells is sufficient to enhance ErbB2 mediated mitogenesis (Tan, Rouen et al. 2003). Furthermore, forced down-regulation of Caveolin-1 in myelinated primary Schwann cell-DRG neuron co-cultures is sufficient to increase ErbB2 mediated demyelination (Yu, Rouen et al. 2008). These results suggest that the loss of Caveolin-1 influences ErbB2 signaling. More specifically, these observations imply that Caveolin-1 is an endogenous regulator of ErbB2 activity. This idea is not a new one, as previous reports suggest that Caveolin-1 expression inversely correlates with ErbB2 (Engelman, Lee et al. 1998; Park, Kim et al. 2005). It's possible that under normal conditions, Caveolin-1 interacts with ErbB2 in a way that prevents unnecessary activation of the receptor. If Caveolin-1 levels decrease though,

receptor activation increases. The evidence described in the above studies is key in demonstrating that Caveolin-1 can have an influence on NRG-1/ErbB2 signaling.

In order for hyperglycemia to have an impact on NRG-1/ErbB2 signaling in a way that impedes nerve function, there would have to be evidence showing hyperglycemia decreases Caveolin-1 expression or function. Such changes have been shown to take place in cultured cells as well as in a mouse model of diabetes (Dobrowsky, Rouen et al. 2005; Hayashi, Juliet et al. 2007). After nine weeks of streptozotocin induced hyperglycemia, mice demonstrated a significant reduction in Caveolin-1 expression in the sciatic nerves (Dobrowsky, Rouen et al. 2005). This decrease in expression was initially observed in immunofluorescently labeled sciatic nerve cross-sections and was confirmed by quantification of mRNA levels (Dobrowsky, Rouen et al. 2005). These observations substantiate the idea that hyperglycemia affects Caveolin-1 and provide the final piece of evidence to support the hypothesis that hyperglycemia modifies NRG-1/ErbB2 signaling, which then mediate nerve dysfunction.

1.5.a Hypothesis

Altered neurotrophism is implicated in the development of diabetic peripheral neuropathy because changes in neuronal growth factor signaling correspond with nerve dysfunction. However, restoring neuronal growth factor signaling does not adequately restore nerve function. The aim of this work is to determine whether or not hyperglycemia induced changes in Schwann cell growth factor signaling also contribute to nerve dysfunction. Neuregulin-1 is the primary growth factor in Schwann cells and signals through the ErbB2 family of receptors. It has been shown that hyperglycemia affects both neuregulin-1 and ErbB2. This influence is most likely indirect and may be at least partially

mediated by Caveolin-1. Therefore, the overall hypothesis for these studies is that the absence of Caveolin-1 would increase ErbB2 activity during hyperglycemia and promote a more rapid or severe neuropathy in myelinated nerves. The specific aims for these studies are:

1. To determine if the loss of Caveolin-1 influences the rate of onset and development of diabetic peripheral neuropathy.
2. To determine if the loss of Caveolin-1 corresponds with an increase in ErbB2 activity.
3. To establish whether or not increased ErbB2 activity in the absence of hyperglycemia is sufficient to induce a neuropathic phenotype.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.a Animals

All animals were housed in the Animal Care Unit at the University of Kansas in accordance with Institutional Animal Care and Use Committee guidelines. Animals were on a twelve-hour light/dark cycle in rooms maintained at 70°C and 70% humidity. Additionally, animals were given ad libitum access to Purina Diet 5001 Rodent Chow and water.

The animals used in these studies include wild-type C57 Bl/6 and Caveolin-1 knockout mice (Cav-1^{-/-}), which were obtained from Harlan Laboratories (Indianapolis, IN) and Jackson Laboratories (Bar Harbor, ME) respectively, and maintained as inbred colonies. The absence of Caveolin-1 was confirmed by genotyping according to Razani *et al.* and immunoblot analysis of lung and sciatic nerve tissue (see Figure 2.1) (Razani, Engelman et al. 2001).

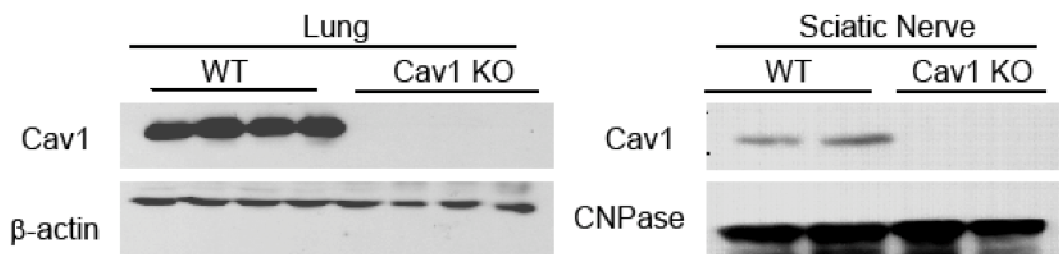
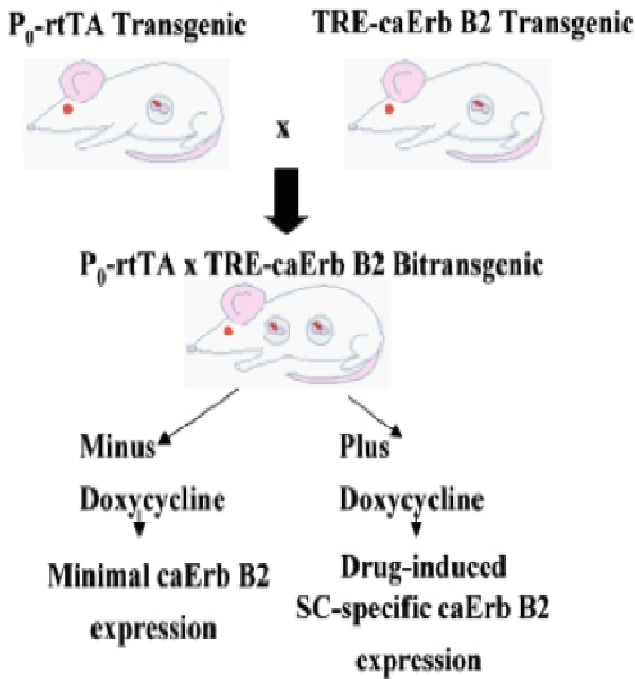


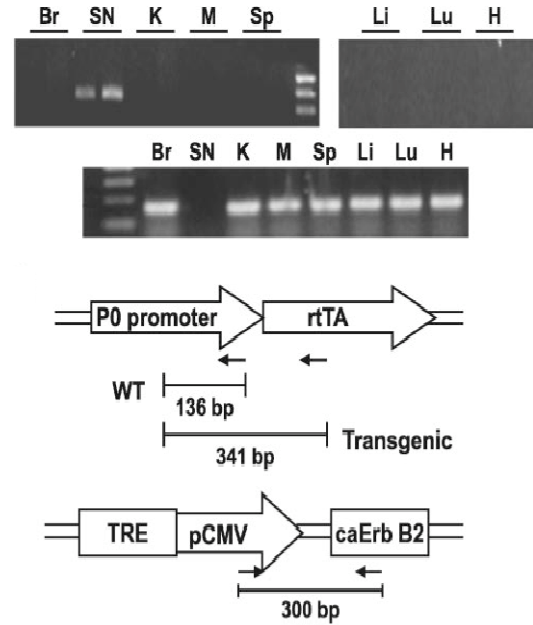
Figure 2.1: Confirmation of the absence of Caveolin-1. Immunoblot analysis on sciatic nerve and lung tissue from wild-type and Cav-1^{-/-} mice was performed in order to confirm the absence of Caveolin-1.

A conditional double transgenic mouse line utilizing the tetracycline inducible system was also used. A detailed description of the generation of the P₀rtTA x caErbB2 mice can be found in McGuire *et al.* (McGuire, Rouen et al. 2009). Briefly though, transgenic mice that broadly express a constitutively active form of ErbB2 (caErbB2, V664 – E664 mutation) under control of the minimal cytomegalovirus promoter and tetracycline response element (TRE) were crossed with a transgenic mouse line expressing the reverse tetracycline transactivator (rtTA) complex gene under control of the rat P₀ promoter. Since expression of P₀ is limited to mature Schwann cells, putting the rtTA gene under control of this promoter minimizes its expression (Messing, Behringer et al. 1992). Bitransgenic identity was confirmed via genotyping, where bitransgenic mice contained the P₀ promoter, the rtTA complex, and the caErbB2 gene (McGuire, Rouen et al. 2009). Expression of the constitutively active form of ErbB2 was induced by ad libitum access to standard rat chow containing 2 mg/kg doxycycline (Bio-Serv Frenchtown, NJ). A diagram summarizing the generation of these bitransgenic mice and the induction of constitutively active ErbB2 expression can be found in Figure 2.2.

A



B



C

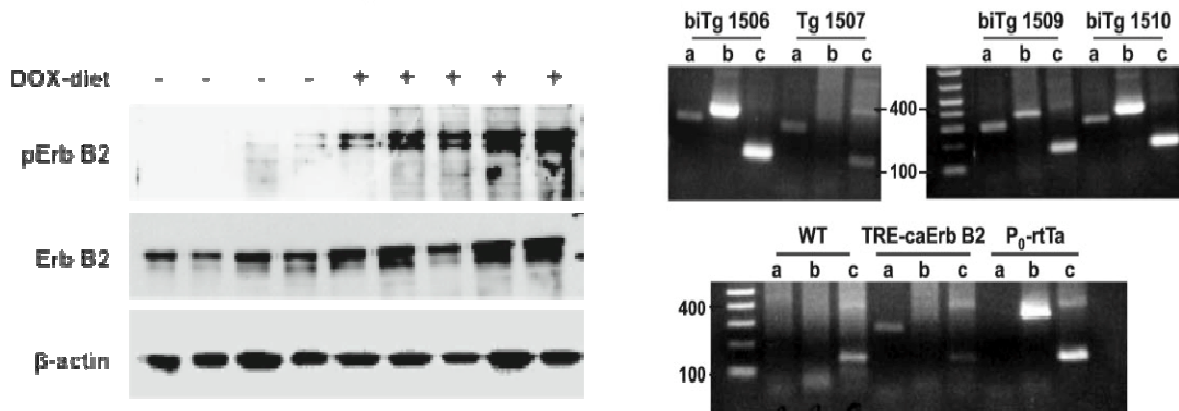


Figure 2.2: Schematic Representation of the Generation and Validation of the P₀rtTA x caErbB2 Double Transgenic Mice. A: Transgenic mice containing the rtTA complex under control of the P₀ promoter were crossed with transgenic mice containing the constitutively active ErbB2 gene and the tetracycline response element under control of the minimal cytomegalovirus promoter. A portion of the offspring of this crossing would then express a constitutively active form of ErbB2 in myelinating Schwann cells when given the tetracycline analog doxycycline. B: Verification of double transgenic status. Double transgenic contain both transgenes as well as the wild-type P₀ promoter, and are positive for the ErbB2 element in sciatic nerve samples only. C: Treatment of double transgenic mice with doxycycline induces an increase in ErbB2 activation without modifying ErbB2 levels.

2.1.b Antibodies

The primary and secondary antibodies used in the studies of this dissertation are listed in Table 2.1. Specific applications employing these antibodies will be described below.

Table 2.1: Antibodies used for various experiments listed below.

Antibody	Manufacturer	Catalog Number
β -actin	Santa Cruz Biotechnology	sc-47778
Caveolin-1	BD Transduction Laboratories	610494
CNPase	Sternberger Monoclonals	SMI-94
ErbB2	Millipore	04-291
TRITC Caveolin-1	Santa Cruz Biotechnology	sc-894 TRITC
MBP	Abcam	Ab53439
NF-H	Santa Cruz Biotechnology	Sc-22909
P ₀	Chemicon	AB5392
pNeu - Tyr 1248 (pErbB2)	Santa Cruz Biotechnology	sc-12352-R
PGP 9.5	Ultraclone	95101
PGP 9.5	AbD Serotec	7863-0504
Goat anti Rabbit HRP	Santa Cruz Biotechnology	sc-2004
Goat anti Mouse HRP	Santa Cruz Biotechnology	sc-2005
Goat anti Rabbit Alexa 488	Molecular Probes	A11008
Goat anti Rabbit Alexa 568	Molecular Probes	A11004
Chicken anti Goat Alexa 647	Molecular Probes	A21469

2.2 Methods

2.2.a Induction of Diabetes

Diabetes was induced in eight-week old mice using the glucose analog streptozotocin (STZ; see Figure 2.3) (Sigma Aldrich, St. Louis, MO). Since STZ competes with glucose for GLUT2 mediated transport into the pancreas, mice were fasted over night prior to receiving STZ. Freshly prepared STZ was administered intraperitoneally (ip) daily at doses of 85 mg/kg, 70 mg/kg, and 55 mg/kg in 200 μ L of sodium

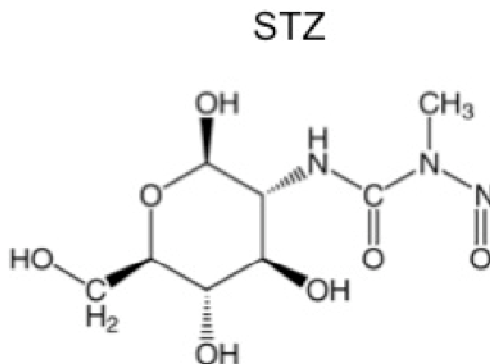


Figure 2.3: Chemical structure of streptozotocin.

citrate/sodium chloride vehicle. Food was withheld for two hours following STZ injection as well. Three days following the final STZ injection, fasting plasma glucose was measured with a One Touch II glucose meter (Lifescan, Milpitas, CA). Animals with a fasting plasma glucose concentration ≥ 290 mg/dL (16 mmol/L) were considered diabetic. Fasting plasma glucose was also measured immediately before euthanasia to confirm diabetic status. Additionally, fasting plasma glucose concentrations were measured every ten weeks during the long-term hyperglycemia study.

2.2.b Measurement of Glycated Hemoglobin

In order to assess glycemic status of mice in long-term studies, percent glycated hemoglobin (Hb A1C) in a blood sample was measured prior to euthanasia. Hb A1C was measured using the A1C Now+ kit (Bayer Healthcare, Sunnyvale, CA). Hyperglycemic status was defined as $> 6\%$ Hb A1C levels.

2.2.c Assessment of Thermal Sensitivity

Sensitivity to thermal stimulation was assessed using the Hargreaves Analgesiometer (Hargreaves, Dubner et al. 1988). Mice were placed unrestrained on the glass surface of the apparatus and allowed to acclimate for about twenty minutes before initiating testing. Activation of the heat source produced a focal, radiant heat that increased in intensity at a rate of approximately 0.3°C/s . The predominant population of sensory fibers activated by such a rate of increase are the C fibers (Yeomans and Proudfit 1996). The heat source was placed under the plantar surface of alternating hind paws and activated at intervals of approximately five minutes. Withdrawal latencies (in seconds) from three to five trials per animal were recorded and averaged.

2.2.d Assessment of Mechanical Sensitivity

Sensitivity to mechanical stimulation was evaluated using the Dynamic Plantar Anesthesiometer (Ugo Basile, Comerio, Italy). This test is based on the use of Von Frey monofilaments to assess A δ fiber function, but instead uses a stiff, 0.5 mm diameter steel monofilament attached

to a force actuator that controls the amount of force produced (Julius and Basbaum 2001; Calcutt 2002). The force actuator was calibrated at the beginning of each assessment session.

Preliminary studies showed that normoglycemic wild-type and Cav-1^{-/-} mice

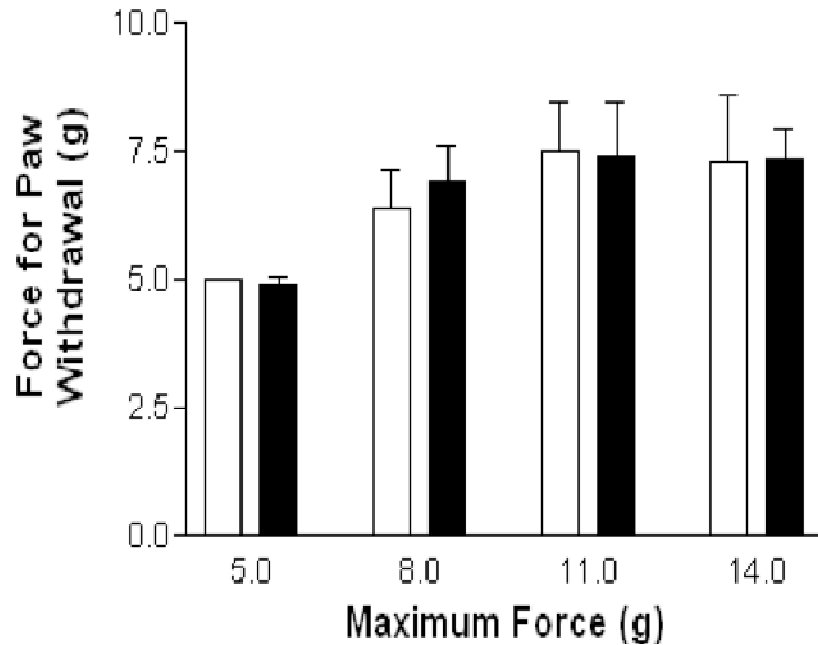


Figure 2.4: Paw Withdrawal at Various Forces. The force required for paw withdrawal differs depending on the maximum force applied. At a maximum force of 5.0 grams, few mice whether Cav-1^{-/-} (black bars) or wild type (white bars), respond at a force below the maximum. At 8.0 grams or above, all animals respond below 7.5 grams suggesting a maximum force of 8.0 would be sufficient to observe changes in mechanical sensitivity induced by hyperglycemia.

were most responsive to forces between 6.0 and 8.0 grams with a ramping speed of 2 seconds (see Figure 2.4). Mice were placed on the wire mesh platform of the apparatus and allowed to acclimate for approximately thirty to forty minutes. After becoming acclimated, the monofilament was placed under the plantar surface of alternate hind paws at intervals of about five minutes. Withdrawal latencies and the force that elicited paw withdrawal from three to five trials per mouse were recorded and averaged.

2.2.e Nerve Conduction Velocity

Equipment

All nerve conduction velocity (NCV) testing was performed using the TECA™ Synergy N2 (Carefusion, San Diego, CA) system and 12 mm subdermal disposable platinum/iridium bipolar needle electrodes (Cardinal Health Neurocare, Madison, WI). Body temperature of each mouse was monitored using a rectal probe and Physitemp TCAT-2DF Controller (Physitemp Instruments, Clifton, NJ), and maintained within one degree of 37°C with a heat lamp.

Anesthesia

Prior to initiating NCV testing, mice were anesthetized with 75 mg/kg Nembutal or a mix of 100 mg/kg Ketamine and 10 mg/kg Xylazine. Anesthesia was confirmed by evaluating the eye blink reflex, where testing was not initiated until failure of the eye blink reflex occurred.

Motor Nerve Conduction Velocity

Motor nerve conduction velocity (MNCV) was assessed by stimulating proximally at the sciatic notch and distally at the ankle with a surpamaximal (9.9 mA) 0.05 ms duration square wave pulse. Resulting waveforms were filtered with low and high settings of 3 and 10 kHz. Compound muscles action potentials were recorded from the first interosseous muscle of the hind paw, where latency was defined as the time from stimulus artifact to onset of negative M-wave deflection. NCV was calculated by dividing the difference in proximal and distal latencies by the distance between stimulating and recording electrodes. Three MNCV values were calculated for each mouse and then averaged.

Sensory Nerve Conduction Velocity

Sensory nerve conduction velocity (SNCV) was evaluated in the hind limb by a 0.05 ms square wave pulse at the second digit using the lowest current that produced maximal response, typically 2.4 -3.0 mA. The waveforms that resulted from such stimulation were again filtered with low and high settings of 3 and 10 kHz. Ten sensory nerve action potentials were recorded from behind the medial malleolus and averaged. Latency for the averaged waveform was determined by the time from stimulus artifact to the onset of peak negative deflection. NCV was calculated by dividing the latency by the distance between stimulating and recording electrodes.

2.2.f PKI 166 Administration

The ErbB2 inhibitor 4-(R)-phenethylamino-6-(hydroxyl) phenyl-7H-pyrrolo[2.3-day]-pyrimidine (PKI 166) was provided by Novartis Institute for Biomedical Research (Basel, Switzerland) (See Figure 2.5). Mice treated with PKI 166 were injected with 25 mg/kg PKI 166 biweekly for the duration of the study. PKI 166 was dissolved in a 10% DMSO and 0.05% TWEEN-80 vehicle and administered ip.

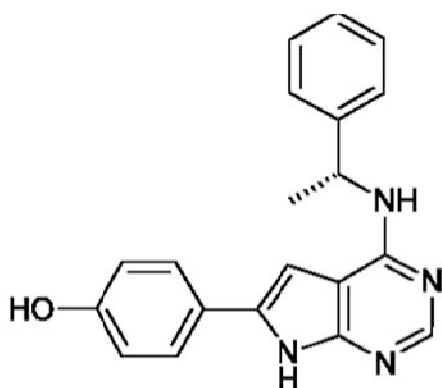


Figure 2.5: Chemical structure of 4-(R)-phenethylamino-6-(hydroxyl) phenyl-7H-pyrrolo[2.3-day]-pyrimidine (PKI 166).

2.2.g Erlotinib Administration

The ErbB2 inhibitor N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine (erlotinib) was provided by OSI Pharmaceuticals (Melville, NY) (See Figure 2.6). Mice treated with erlotinib were injected with 3.12, 6.25, 12.5, or 25 mg/kg erlotinib biweekly for the duration of the study. Erlotinib was dissolved in 6% Captisol (CyDex Pharmaceuticals, Lenexa, KS) and administered ip.

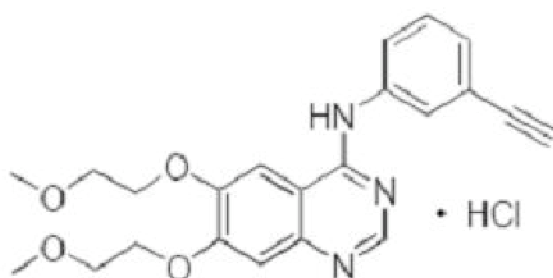


Figure 2.6: Chemical structure of N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine (Erlotinib).

2.2.h Immunofluorescence Analysis

Footpads

The integument of the plantar surface of both hind paws, including the footpads, was dissected and fixed in Zamboni's fixative (3% paraformaldehyde, 15% picric acid) (Newcomer Supply, Middleton, WI) overnight. Tissues were then rinsed and stored in PBS with sodium azide at 4°C. Prior to freezing for the preparation of frozen sections, tissues were incubated in 30% sucrose overnight. Tissues were then embedded in Tissue-Tek optimal cutting temperature compound (OCT) (Sakura USA, Torrence, CA), frozen on dry ice, and stored at -80°C until being sectioned. Frozen tissues were sectioned at 30 µm and placed on Fisherbrand Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA).

Immunofluorescence of footpad sections was performed as described (Johnson, Ryals et al. 2008). Briefly though, footpad tissue was surrounded by PAP pen (Newcomer Supply,

Middleton, WI), incubated in a blocking solution (0.5% porcine gelatin, 1.5% normal goat serum, 0.5% Triton X-100 in Superblock Buffer [Peirce, Rockford, IL]) for one hour at room temperature. Slides were then incubated with a 1:400 dilution of an antibody against the pan-neuronal marker PGP 9.5 (Ultraclone, Isle of Wight, UK) overnight at 4°C. Slides were then rinsed in PBS, incubated with fluorescently labeled secondary antibody (goat anti-rabbit Alexa 555; 1:2000 dilution) (Molecular Probes, Eugene, OR), rinsed in PBS, and then stored at 4°C.

Non-myelinated C-fibers in the epidermis of footpad tissue were then viewed by the excitation and emission of the fluorescently labeled secondary antibody. Intra-epidermal nerve fiber density (IENF) was quantified by counting all fluorescently labeled fibers that crossed the dermal/epidermal junction of three fields of view per footpad section. Four footpad sections on two separate slides for each animal were analyzed. The distance in millimeters of each field of view was also measured so that IENF could be reported in the number of fibers per mm.

Sciatic Nerve Cross Sections

Sciatic nerves were dissected immediately following euthanasia and flash frozen in liquid nitrogen. Frozen sciatic nerve transverse sections were prepared as described (Tan, Rouen et al. 2003). The University of Kansas Medical Center Histology Facility performed the embedding and sectioning of sciatic nerve tissue to be used for immunofluorescence analysis. Slides were stored at -80°C until staining.

Frozen sections were brought to room temperature and fixed in freshly prepared 4%paraformaldehyde/2% glutaraldehyde for 45 minutes. Slides were then rinsed in PBS and incubated with blocking solution (5% normal goat serum in PBS) for 1 hour at room

temperature. Next, slides were incubated with a 1:100 dilution of pErbB2 antibody overnight at 4°C, rinsed in PBS, and incubated with Alexafluor 488 labeled goat anti-rabbit secondary antibody (1:2000) for 2 hours at room temperature. Slides were then rinsed in PBS and incubated with a 1:400 dilution of anti-neurofilament-H antibody overnight at 4°C. Again, slides were rinsed with PBS and then incubated with Alexafluor 647 labeled chicken anti-goat secondary antibody (1:2000) for 2 hours at room temperature. Finally, slides were rinsed in PBS and incubated in a 1:400 dilution of FITC conjugated anti-Caveolin-1 antibody. Slides were rinsed in PBS, coverslipped, and stored until viewing. Immunolabeled sciatic nerve cross sections were imaged using an Olympus spinning disk confocal microscope (Olympus, Center Valley, PA) with the Slidebook software package (3i, Denver, CO).

Teased Sciatic Nerve

Sciatic nerves were dissected immediately following euthanasia and fixed in freshly prepared 4%paraformaldehyde/2% glutaraldehyde overnight. After fixation, nerves were placed in PBS with sodium azide until teasing. Using 1 µm diameter tungsten needles (Fine Science Tools, Foster City, CA) teasing of sciatic nerves was performed according to Guertin *et al.* (Guertin, Zhang et al. 2005). Teased nerves were placed on Fisherbrand Superfrost Plus microscope slides and stored at 4°C until being immunofluorescently labeled.

Labeling of teased nerves was done in a similar manner to the sciatic nerve cross sections described above. Slides were incubated in blocking solution for 1 hour at room temperature, rinsed in PBS, and then incubated in a 1:200 dilution of anti pErbB2 antibody overnight at 4°C. Next, slides were rinsed in PBS and incubated in Alexafluor 568 labeled goat anti rabbit secondary antibody (1:2000 dilution). Finally, slides were rinsed in PBS,

incubated in a 1:400 dilution of anti Ezrin antibody overnight at 4°C, and rinsed in PBS again. Slides were coverslipped and stored at 4°C until being imaged. Imaging was performed in the same fashion as were the sciatic nerve cross sections.

2.2.i Immunohistochemistry of Footpad Tissue

Preparation of footpad tissue for immunohistochemical analysis was performed in a similar fashion to that described above for immunofluorescence analysis. The integument of the plantar surface of both hind paws was dissected and fixed in Zamboni's fixative overnight. Tissues were then rinsed and stored in PBS with sodium azide at 4°C. Prior to sectioning, tissues were cryoprotected in 30% sucrose overnight, embedded in OCT, frozen on dry ice, and stored at -80°C. Frozen tissues were sectioned at 30 µm, placed on Fisherbrand Superfrost Plus microscope slides, and stored at -80°C until immunohistochemistry was performed.

Immunohistochemistry was performed using the Vectastain Elite ABC-Peroxidase kit for rabbit IgG (Vector Laboratories, Burlingame, CA) and an anti PGP 9.5 antibody (AbD Serotec, Raleigh, NC). Briefly, slides were incubated in blocking buffer containing normal goat serum for 30 minutes, incubated in a 1:1000 dilution of anti-PGP 9.5 antibody for three hours at room temperature, rinsed in PBS, and then incubated with provided secondary antibody for 1 hour at room temperature. Slides were then rinsed in PBS, incubated in the provided avidin-biotin complex solution (ABC solution) for 1 hour at room temperature, rinsed in PBS, and then incubated in NovaRED peroxidase substrate solution (Vector Laboratories, Burlingame, CA) for 2-3 minutes. Sections were then counterstained with hematoxylin and coverslipped.

Slides were imaged using a Zeiss Axioplan-2 light microscope (Carl Zeiss Microimaging, Thornwood, NY) and a color ccd digital camera (Diagnostic Instruments Inc., Sterling Heights, MI). Two slides from each animal were stained, and three images from two sections on each slide were captured, for a total of twelve images per animal.

Immunopositive profiles corresponding to nerve fibers crossing the dermal/epidermal junction were quantified from each image. The length of the dermal/epidermal junction was also measured so that the number of fibers per image could be normalized to length.

2.2.j g-ratio Quantification

Sciatic nerves were dissected immediately following euthanasia and fixed in freshly prepared 4% paraformaldehyde/2% glutaraldehyde overnight at 4°C. Nerves were then rinsed with PBS and stored at 4°C in PBS containing sodium azide until embedding was to take place. Nerves were either epon embedded at the University of Kansas Medical Center Electron Microscopy lab or embedded in OCT compound for frozen sectioning. Transverse sections of epon embedded sciatic nerves were cut at 3 µm and stored at room temperature until staining. Transverse frozen sections, on the other hand, were cut at 5 µm, placed on Fisherbrand Superfrost Plus microscope slides, and stored at -80°C until being staining with toluidine blue.

Toluidine staining was performed following a protocol from the University of Kansas Medical Center Electron Microscopy lab. Briefly, slides, whether from epon embedded or frozen sections, were placed on a hot plate on the lowest setting and incubated in a 1% toluidine blue solution for about 30 seconds. Slides were then rinsed in ddH₂O, blotted with a kimwipe to remove excess water, and then incubated in an acid alcohol

solution (1% HCl, 50% EtOH) for 45 seconds. Finally, slides were rinsed in ddH₂O, dried on a hotplate, and coverslipped. Slides were then incubated at 70°C for approximately 24 hours.

Stained slides were then imaged on the same Zeiss light microscope as the immunohistochemically stained footpads. The number of images taken per slide was dependent on the group of animals being analyzed, but was generally between 3-5 images per slide and two slides per animal. From these images, the g-ratio, or ratio of axon diameter to fiber diameter (axon plus myelin sheath; see Figure 2.7) was quantified for approximately 1000 axons per treatment group using ImageJ software. g-ratio quantification using ImageJ requires the measurement of axonal and fiber diameter twice for each fiber. The mean value of axonal and fiber diameter was then calculated and used to derive the g-ratio. When quantifying several treatment groups, this method is time consuming, tedious, and prone to subjective bias. In an attempt to cut down on time required for quantification and to decrease subjectivity in the measures, we used the CellProfiler software package to automate the quantification of the g-ratio. CellProfiler identifies axonal and myelin regions and performs myriad measures of these two regions including axis lengths and areas (see Figure 2.8). Comparing these parameters provides an alternative and rapid means to accurately quantify the g-ratio in nerve cross sections.

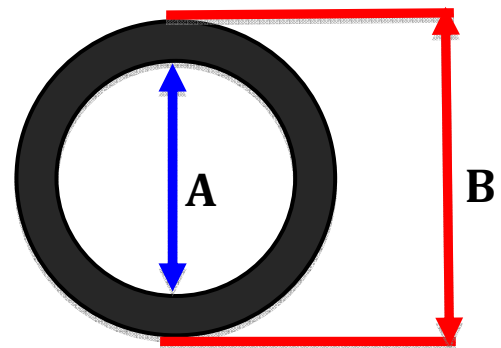


Figure 2.7: Schematic representation of a nerve cross section and g-ratio quantification. g-ratio = A/B.

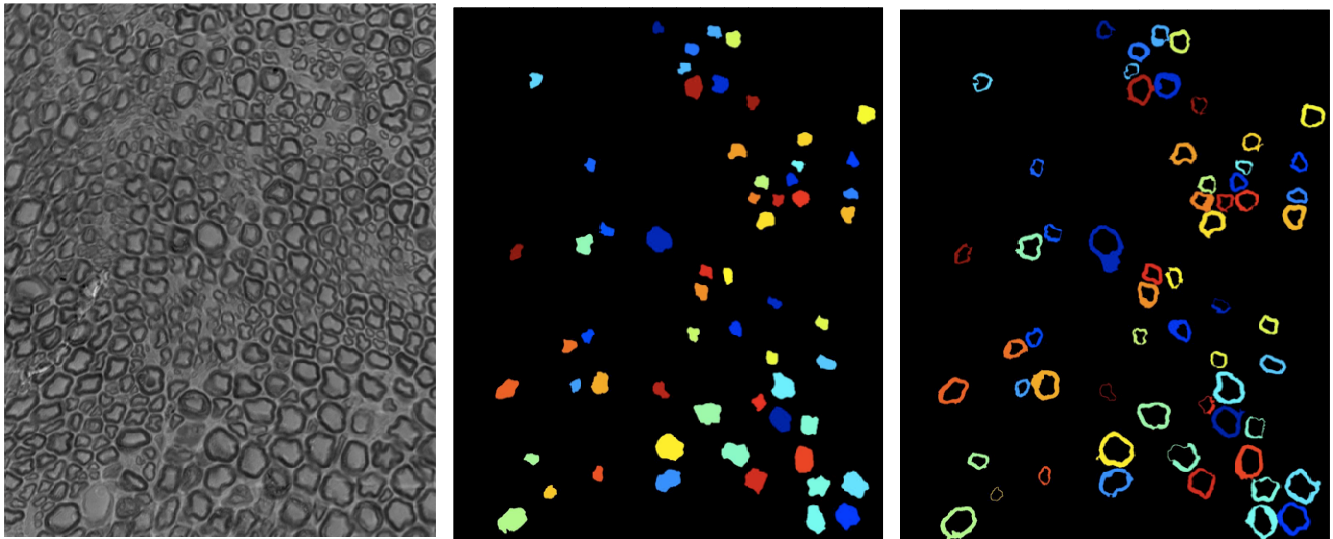


Figure 2.8: Axon and Myelin Sheath Identification in CellProfiler. Cell profiler uses the input image on the left to identify axons (middle) and myelin sheaths (right). After identifying objects in the image, the software measures numerous

2.2.k Immunoblot Analysis

Immunoblot analysis was performed on a mixture of sciatic, tibial, and sural nerves, as well as lung tissue. Tissues were dissected immediately following euthanasia and placed in 0.2 mL of modified radio immunoprecipitation assay (mRIPA) buffer (50 mmol/l Tris-HCl, pH 7.5, 1 mmol/l EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 150 mmol/l NaVO₃, 0.5 mmol/l sodium molybdate, 40 mmol/l NaF, 10 mmol/l α -glycerophosphate, and 1X Complete Protease inhibitors [Roche Diagnostics]) and homogenized with a Polytron fitted with a micro tissue tearor. Cellular debris was sedimented by centrifugation at 10,000 x g for 10 minutes at 4°C, and the protein concentration of the supernatant was determined. Twenty μ g of protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane for immunoblot analyses.

After transfer of proteins to nitrocellulose membranes was complete, nitrocellulose membranes were incubated in blocking solution (5% milk in PBS-T) for two hours at room

temperature with gentle rocking. Membranes were then incubated with primary antibody overnight at 4°C (except for membranes incubated with anti β -actin, which were incubated for 3 hours at room temperature). After incubation with primary antibody, membranes were washed in blocking buffer and then incubated with secondary antibody for three hours at room temperature. Membranes were then washed in PBS-T and incubated for approximately 5 minutes with a HRP conjugated chemiluminescence detection kit (Amersham Biosciences). Film was exposed to the resulting chemiluminescent signal and developed. Immunoblots were quantified using densitometry of developed films with the aid of ImageJ software. Levels of the protein of interest were normalized to levels of β -actin and reported as a percent of the value observed in control subjects.

2.2.1 Data Analysis

Data are presented as arithmetic means \pm SE. After verifying equality of variances, difference between treatment and genotype groups were determined with factorial ANOVAs. When ANOVAs determined the presence of significant differences, specific differences were determined using the Tukey HSD posthoc test. All statistical analyses were performed using Systat 12 (Systat Software, Chicago, IL).

CHAPTER 3: RESULTS

3.1 Hyperglycemia and the Absence of Caveolin-1

In order to understand how the absence of Caveolin-1 influences the rate of onset and development of diabetic peripheral neuropathy, we induced diabetes in Caveolin-1^{-/-} and wild-type mice and evaluated nerve function and several morphological parameters associated with nerve function. The induction of diabetes using STZ resulted in a three- to fourfold increase in fasting plasma glucose levels (Table 3.1). The generation of diabetes also led to the reduction in weight gain in both Cav-1^{-/-} and wild-type mice.

Table 3.1: Fasting plasma glucose and weights for diabetic and non-diabetic Cav-1^{-/-} and wild-type mice. Mean \pm SD.

Duration of diabetes (weeks)	Cav 1 ^{-/-} STZ			Cav 1 ^{-/-} V		
	BG (mg/dL)	Weight (g)	N	BG (mg/dL)	Weight (g)	N
1	495 \pm 94.0	19.58 \pm 1.71	8	108 \pm 17.5	22.54 \pm 2.88	8
2	521 \pm 92.9	18.84 \pm 2.23	10	140 \pm 22.5	21.79 \pm 2.76	8
3	552 \pm 60.3	18.71 \pm 3.22	11	123 \pm 26.1	22.50 \pm 2.40	9
4	564 \pm 71.6	22.25 \pm 3.72	12	112 \pm 19.9	23.15 \pm 2.97	8
6	519 \pm 106.7	19.32 \pm 3.32	9	127 \pm 13.4	25.51 \pm 2.42	9
12	594 \pm 11.5	21.24 \pm 3.10	4	179 \pm 30.6	32.22 \pm 1.08	6

Duration of diabetes (weeks)	C57 STZ			C57 V		
	BG (mg/dL)	Weight (g)	N	BG (mg/dL)	Weight (g)	N
1	435 \pm 71.1	20.42 \pm 1.56	7	141 \pm 17.0	21.12 \pm 2.04	6
2	563 \pm 51.9	20.52 \pm 2.05	10	154 \pm 20.2	23.13 \pm 1.99	9
3	531 \pm 83.5	21.24 \pm 1.78	8	139 \pm 23.1	22.10 \pm 2.21	9
4	508 \pm 74.6	21.50 \pm 1.59	10	142 \pm 26.5	22.52 \pm 1.58	10
6	471 \pm 81.0	21.69 \pm 2.69	12	175 \pm 26.1	27.58 \pm 3.89	10
12	512 \pm 113.2	23.60 \pm 2.08	7	141 \pm 21.3	25.61 \pm 3.89	6

After confirmation of diabetic status, nerve function was indirectly assessed with the measurement of withdrawal latency from thermal stimulation (Figure 3.1) and force required for paw withdrawal from mechanical stimulation (Figure 3.2). In both instances, diabetic

Cav-1^{-/-} mice demonstrated a rapid and significant decrease in sensitivity. After one week of hyperglycemia the Cav-1^{-/-} mice developed a significant deficit in thermal and mechanical insensitivity, requiring nearly twice as long and 20% more force to respond. The rapid onset of thermal insensitivity was delayed in wild-type mice, which developed a transient deficit that became chronic as diabetes progressed. Somewhat surprisingly, thermal insensitivity continued to worsen as the duration of diabetes increased. After development of the initial

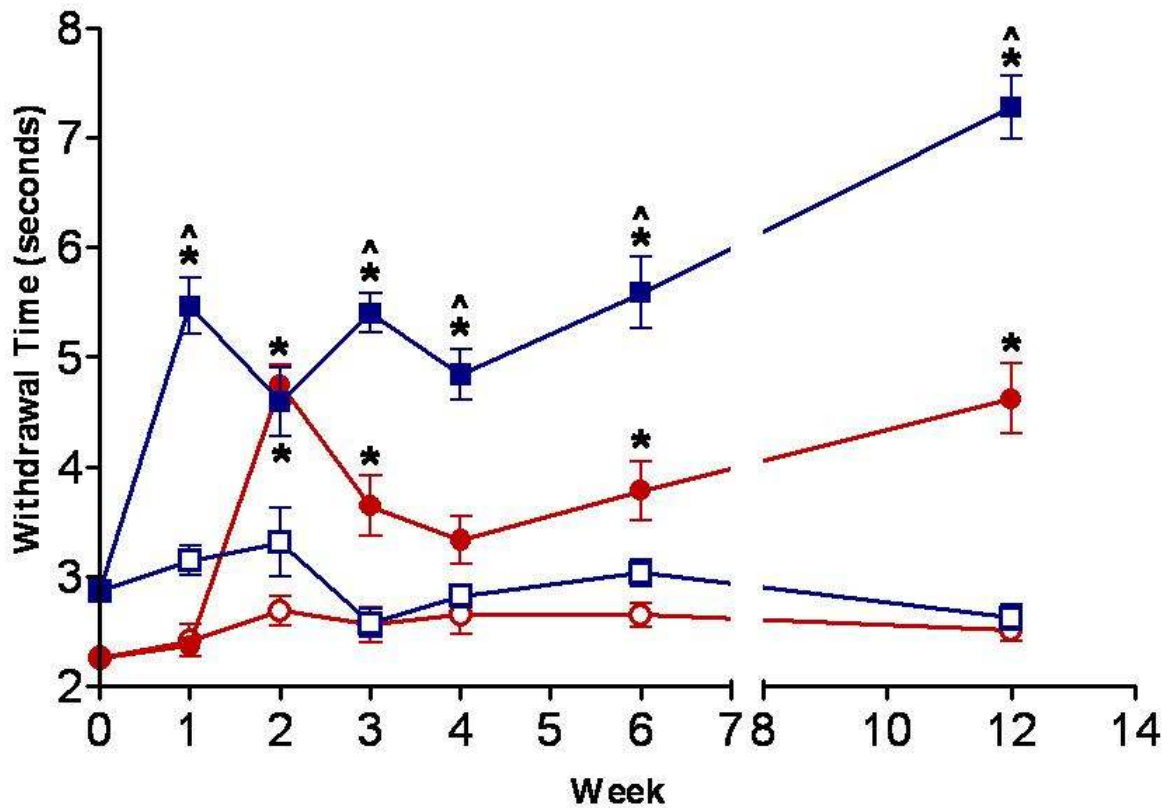


Figure 3.1: Thermal Sensitivity in Cav-1^{-/-} and Wild-type mice over 14 weeks of STZ induced diabetes. Diabetic Cav-1^{-/-} mice (solid blue boxes) demonstrate an earlier and more severe onset of thermal insensitivity than do diabetic wild-type mice (solid red circles) and non-diabetic controls (Cav-1^{-/-}: open blue boxes, wild-type: open red circles). * = $p < 0.05$ for diabetic vs. non-diabetic. ^ = $p < 0.05$ for Cav-1^{-/-} vs. wild-type.

deficit in mechanical sensitivity, Cav-1^{-/-} mice recovered some sensitivity but remained significantly more insensitive than non-diabetic Cav-1^{-/-} mice and diabetic wild-type mice for the remainder of the study. Diabetic wild-type mice also developed a mechanical

insensitivity as a result of STZ induced diabetes. However, there was no rapid onset of insensitivity like the one observed in the Cav-1^{-/-} mice. Rather, the insensitivity developed as the duration of diabetes increased. The absence of Caveolin-1 alone though did not have an impact on nerve function when assessed with these methods.

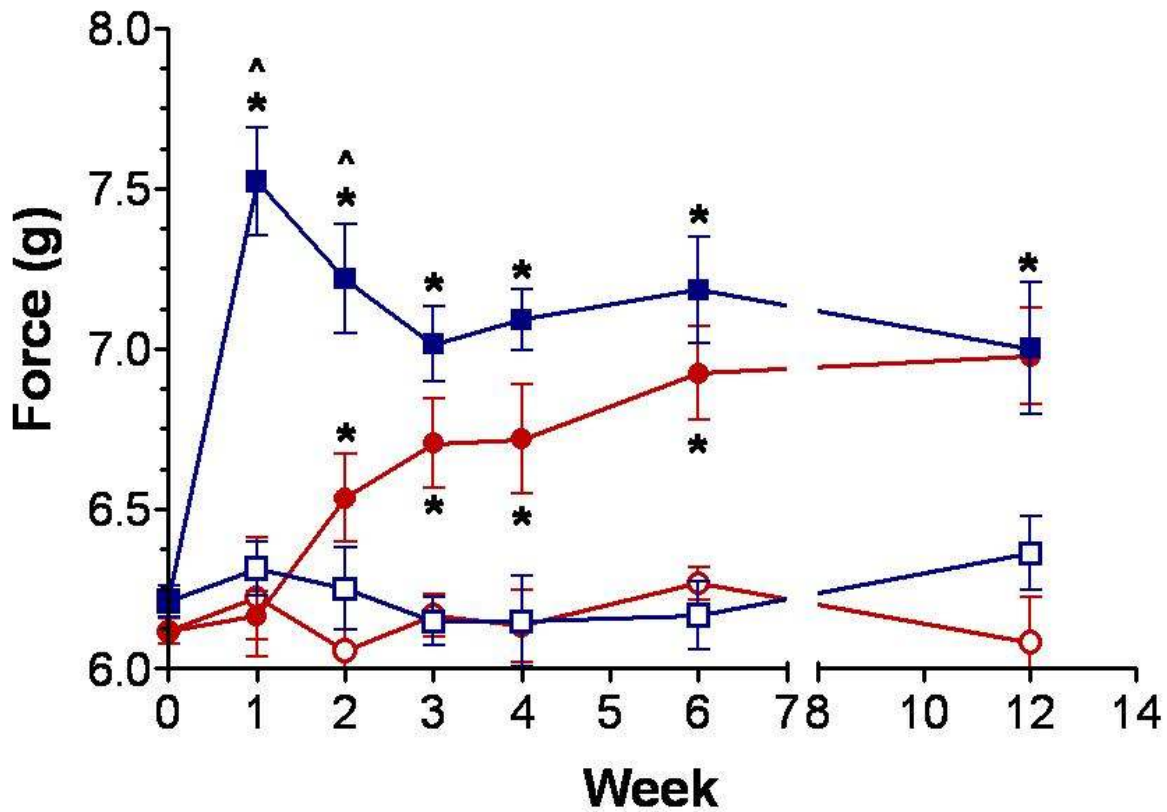


Figure 3.2: Mechanical Sensitivity in Cav-1^{-/-} and Wild-type mice over 14 weeks of STZ induced diabetes. Diabetic Cav-1^{-/-} mice (solid blue boxes) demonstrate an earlier and more severe onset of mechanical insensitivity than do diabetic wild-type mice (solid red circles) and non-diabetic controls (Cav-1^{-/-}: open blue boxes, wild-type: open red circles). * = $p < 0.05$ for diabetic vs. non-diabetic. ^ = $p < 0.05$ for Cav-1^{-/-} vs. wild-type.

In order to evaluate nerve function directly, we measured motor and sensory nerve conduction velocity in diabetic and non-diabetic Cav-1^{-/-} and wild-type mice. Since we observed a rapid onset of mechanical and thermal insensitivity in diabetic Cav-1^{-/-} mice, we

expected nerve conduction velocities to also be significantly reduced after short durations of diabetes. This expectation was fulfilled for MNCV (Figure 3.3) but not for SNCV (Figure 3.4). As early as one week after the onset of hyperglycemia, diabetic Cav-1^{-/-} mice demonstrate a 10 – 15% reduction in MNCV compared to diabetic wild-type mice, and a 20 – 25% decline compared to non-diabetic Cav-1^{-/-} mice. This reduction was consistent

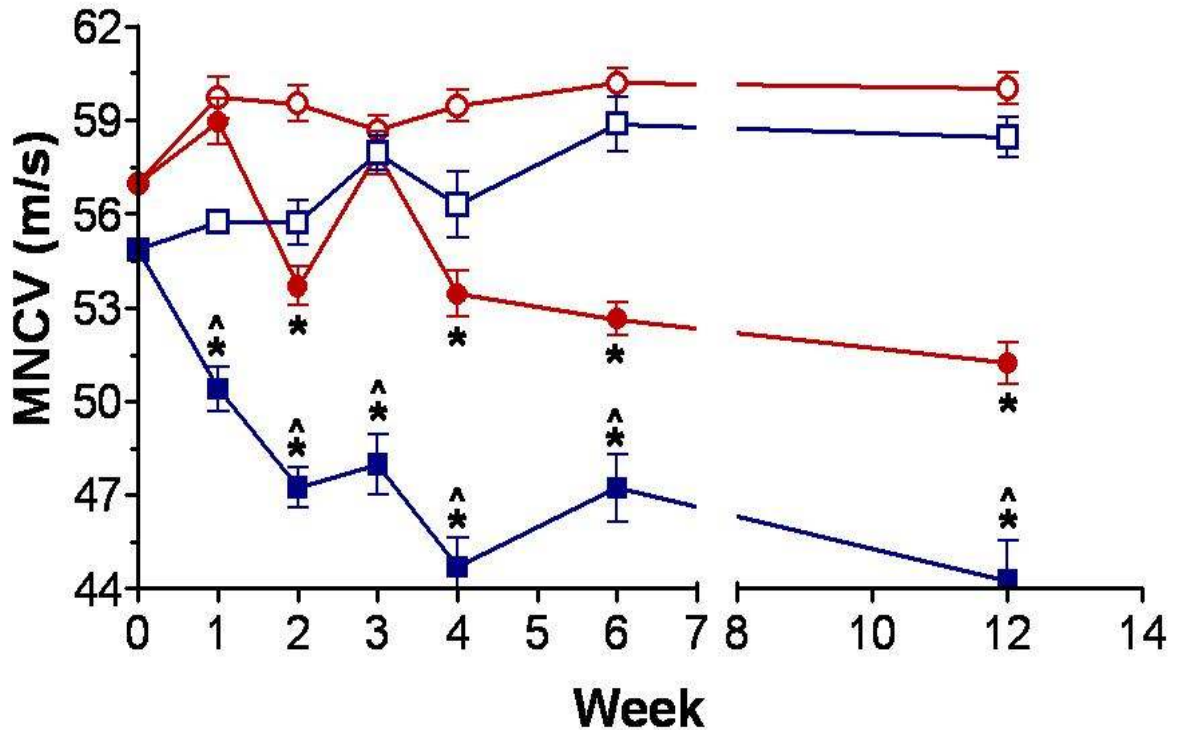


Figure 3.3: Motor Nerve Conduction Velocity in Cav-1^{-/-} and Wild-type mice over 14 weeks of STZ induced diabetes. Diabetic Cav-1^{-/-} mice (solid blue boxes) demonstrate an earlier and more severe onset of deficits in MNCV than do diabetic wild-type mice (solid red circles) and non-diabetic controls (Cav-1^{-/-}: open blue boxes, wild-type: open red circles). * = $p < 0.05$ for diabetic vs. non-diabetic. ^ = $p < 0.05$ for Cav-1^{-/-} vs. wild-type.

throughout the duration of the study as diabetic wild-type mice gradually developed a consistent deficit in MNCV as well. Although we observed deficits in mechanical and thermal sensitivity after short periods of hyperglycemia, we only observed significant reductions in SNCV in diabetic Cav-1^{-/-} mice after 12 weeks of diabetes. It is again

important to note that the greater deficit in MNCV and the eventual development of the SNCV deficit observed in Cav-1^{-/-} mice are not the result of a developmental neuropathy since the absence of Caveolin-1 did not have an affect on motor or sensory nerve conduction velocity. The rapid onset of mechanical insensitivity, thermal insensitivity, and MNCV in diabetic Cav-1^{-/-} mice suggests that the absence of Caveolin-1 influences the rate of onset and development of diabetic peripheral neuropathy involving myelinated nerves.

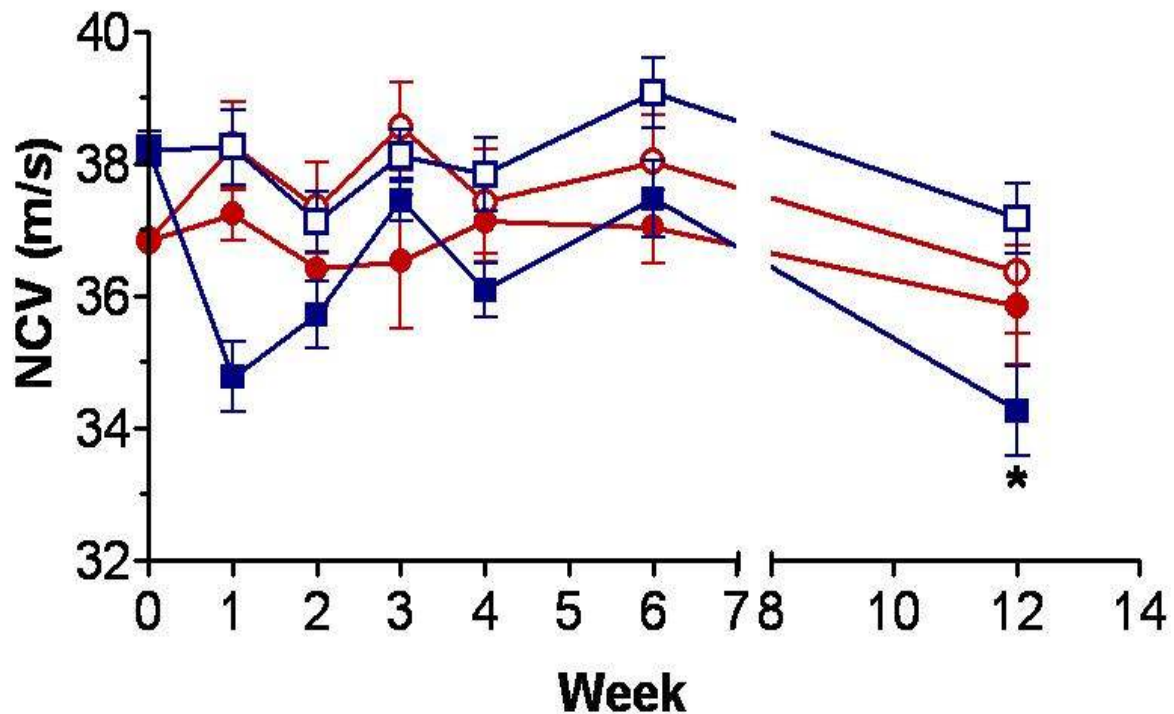


Figure 3.4: Sensory Nerve Conduction Velocity in Cav-1^{-/-} and Wild-type mice over 14 weeks of STZ induced diabetes. Diabetic Cav-1^{-/-} mice (solid blue boxes) do not demonstrate a significant deficit in SNCV compared to their non-diabetic counterparts until 12 weeks of hyperglycemia. Diabetic Cav-1^{-/-} mice also do not demonstrate significant deficits compared to diabetic wild-type mice (solid red circles) at any time period. Additionally, diabetic wild-type mice do not show deficits in SNCV when compared to controls (Cav-1^{-/-}: open blue boxes, wild-type: open red circles). * = $p < 0.05$ for diabetic vs. non-diabetic.

In order to determine whether or not the observed behavioral deficits could have been mediated by changes in nerve innervation of the plantar surface of the paw, we quantified epidermal innervation after two, six, and twelve weeks of diabetes (Figure 3.5).

Intraepidermal nerve fiber density (IENFD) is an increasingly utilized method for assessing the progression of diabetic neuropathy, where increasing duration of diabetes corresponds with a decrease in IENFD. We did not observe any difference between diabetic and non-diabetic Cav-1^{-/-} and wild-type mice at two, six, and twelve weeks when quantifying epidermal innervation. Although we did not detect a difference in IENFD, it is plausible that twelve weeks of diabetes is not sufficient to induce a loss of epidermal nerve fibers.

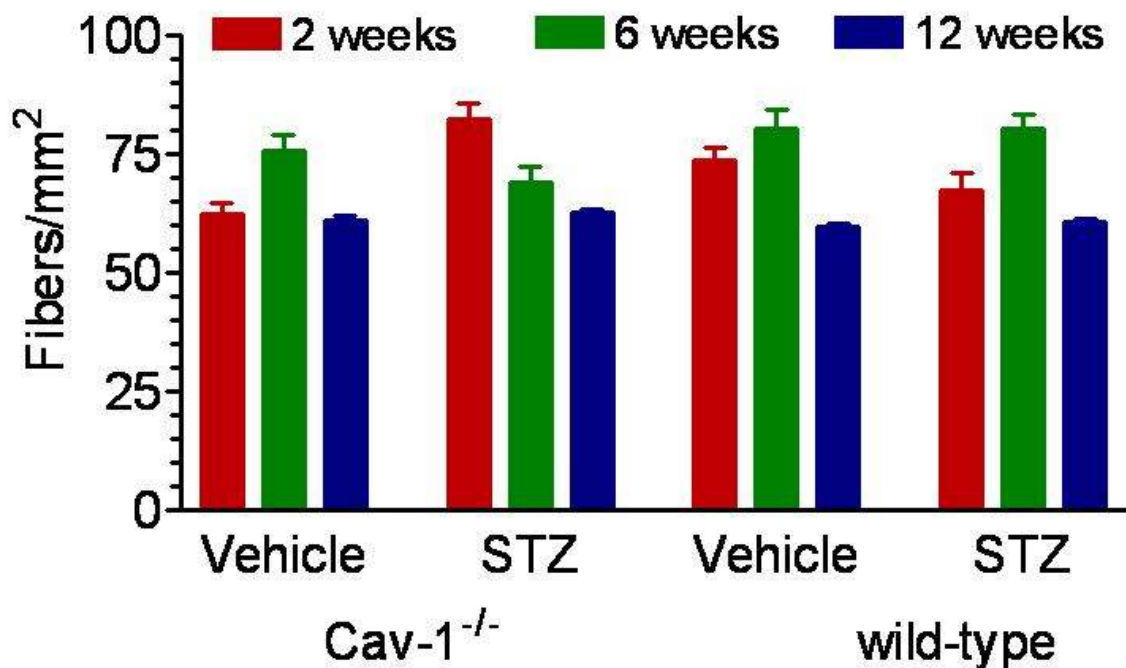


Figure 3.5: Intraepidermal Nerve Fiber Density in Cav-1^{-/-} and Wild-type mice after 2, 6, and 12 weeks of STZ induced diabetes. STZ induced hyperglycemia did not have an affect on IENFD in Cav-1^{-/-} or wild-type mice after 12 weeks of diabetes as no significant differences were observed between any groups.

However, our results do indicate that the deficits in mechanical and thermal sensitivity cannot be attributed to innervation of the plantar surface of the hind paws, where the stimuli for these tests are focused.

Although we observed deficits in mechanical sensitivity, thermal sensitivity, and MNCV, we did not observe a reduction in the expression of the compact myelin protein P₀ (Figure 3.6). After twelve weeks of diabetes, the expression of P₀ in the sciatic nerves of Cav-1^{-/-} and wild-type mice was not different, suggesting the behavioral and electrophysiologic changes that we observed were not a result of a decline in one of the major components of the myelin sheath. Therefore, it is not likely that demyelination is a contributing factor in the nerve dysfunction caused by short durations of hyperglycemia.

We hypothesized that the absence of Caveolin-1 would correspond with an increase in the activation of ErbB2. Therefore, we evaluated ErbB2 activation in sciatic nerve homogenates from diabetic and non-diabetic Cav-1^{-/-} and wild-type mice at two, six, and



Figure 3.6: Expression of P₀ in Cav-1^{-/-} and wild-type mice after 12 weeks of STZ induced diabetes. Cav-1^{-/-} and wild-type mice do not demonstrate significant reductions in expression of the compact myelin protein P₀.

twelve weeks after the induction of diabetes. Recall that the activation of the ErbB2 receptor requires phosphorylation of tyrosine residues. Therefore, ErbB2 activation is detected with an antibody against phosphorylated ErbB2 (pErbB2). Figure 3.7 displays the immunoblot analysis and quantification of ErbB2 activation after two weeks of diabetes. At this duration of diabetes, diabetic Cav-1^{-/-} demonstrated significantly greater activation of ErbB2 than did diabetic wild-type and non-diabetic mice. We observed a fourfold increase in pErbB2 in

diabetic Cav-1^{-/-} mice when compared to control mice. It is important to note that we did not detect any significant changes in the expression of ErbB2 at this time period. After six weeks of diabetes (Figure 3.8), diabetic Cav-1^{-/-} mice demonstrated an even greater increase in ErbB2 activation. At this point, there was a nearly sevenfold increase in the presence of pErbB2. Additionally, diabetic wild-type mice also demonstrated a significant enhancement in ErbB2 activation when compared to non-diabetic mice. Furthermore, amplified ErbB2 activation was also found in sciatic nerve cross sections from wild-type mice after six weeks of diabetes. Figure 3.10 shows the increase in the presence of pErbB2 in cross sections from diabetic animals. ErbB2 activation did not continue to rise in diabetic Cav-1^{-/-} and wild-type mice when evaluated after 12 weeks of diabetes (Figure 3.9). Although diabetic Cav-1^{-/-} mice still demonstrated a fourfold increase in pErbB2 levels compared to non-diabetic control mice, the increase in ErbB2 activity after 12 weeks was similar to that observed after 2 weeks of diabetes and lower than that observed after 6 weeks of diabetes. Furthermore, diabetic wild-type mice no longer demonstrated increased ErbB2 activation at this time period. The reason for this drop in ErbB2 activation from 6 to 12 weeks is unknown, but may represent a compensatory mechanism attempting to restore normal ErbB2 signaling. One interesting finding from these studies was how closely the changes in ErbB2 activation resemble the development of behavioral and electrophysiologic deficits. Diabetic Cav-1^{-/-} mice developed severe deficits in mechanical and thermal sensitivity and MNCV within two weeks of becoming diabetic. These same mice also demonstrated significantly increased ErbB2 activation at this early time point. After six weeks of diabetes, behavioral and electrophysiologic deficits had not gotten worse in diabetic Cav-1^{-/-} mice, but diabetic wild-type mice, which began demonstrating increased ErbB2 activity, also began consistently

demonstrating these behavior and conduction velocity deficits. These data indicate that hyperglycemia increases the phosphorylation of ErbB2 and that the absence of Caveolin-1 enhances hyperglycemia's affect, suggesting that Caveolin-1 is an endogenous regulator of ErbB2.

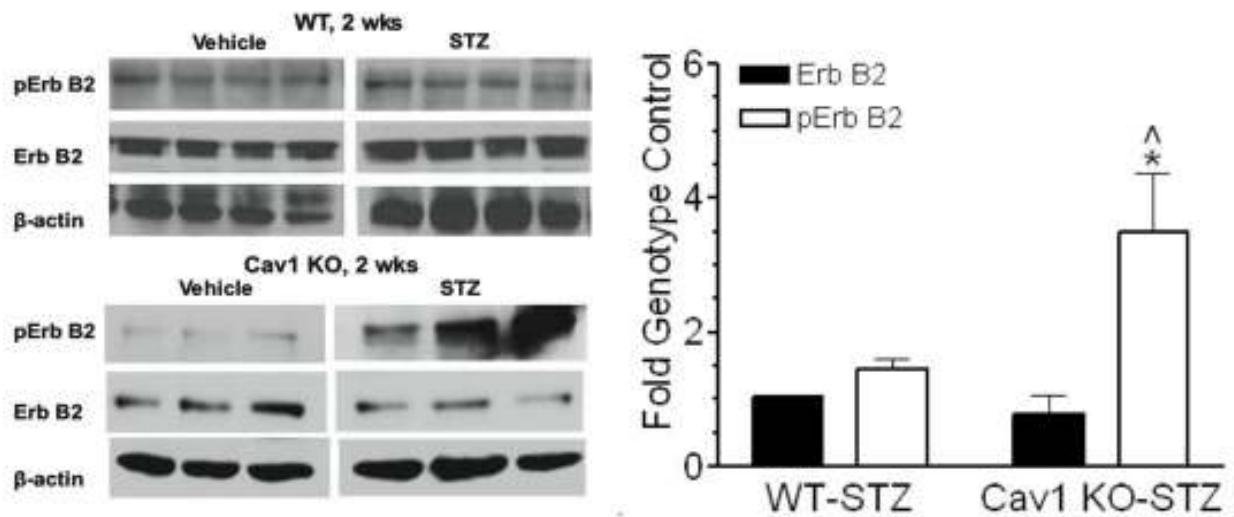


Figure 3.7: ErbB2 activation after 2 weeks of diabetes. Diabetic Cav1^{-/-} mice demonstrate a significant increase in ErbB2 activation, evidenced by an increase in the presence of pErbB2. Levels of pErbB2 and ErbB2 are normalized to actin levels. * = p < 0.05 for diabetic vs. non-diabetic. ^ = p < 0.05 for Cav1^{-/-} vs. wild-type

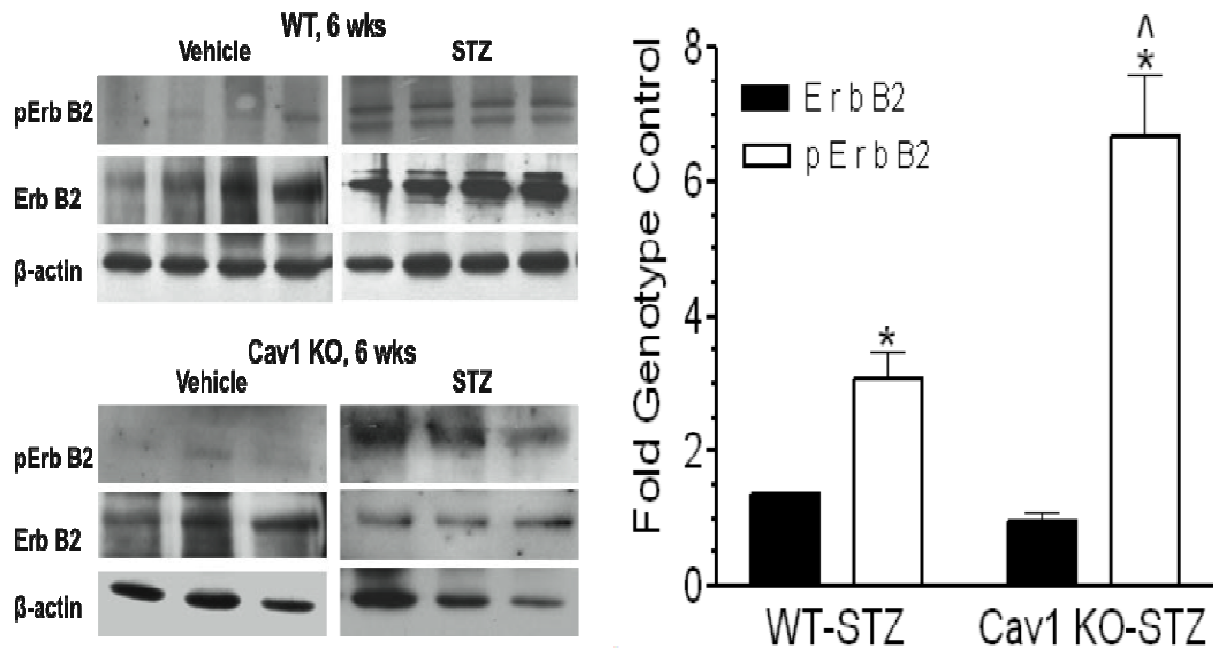


Figure 3.8: ErbB2 activation after 6 weeks of diabetes. Diabetic Cav-1^{-/-} and wild-type mice demonstrate a significant increase in ErbB2 activation, evidenced by an increase in the presence of pErbB2. Levels of pErbB2 and ErbB2 are normalized to actin levels. * = $p < 0.05$ for diabetic vs. non-diabetic. ^Λ = $p < 0.05$ for Cav-1^{-/-} vs. wild-type

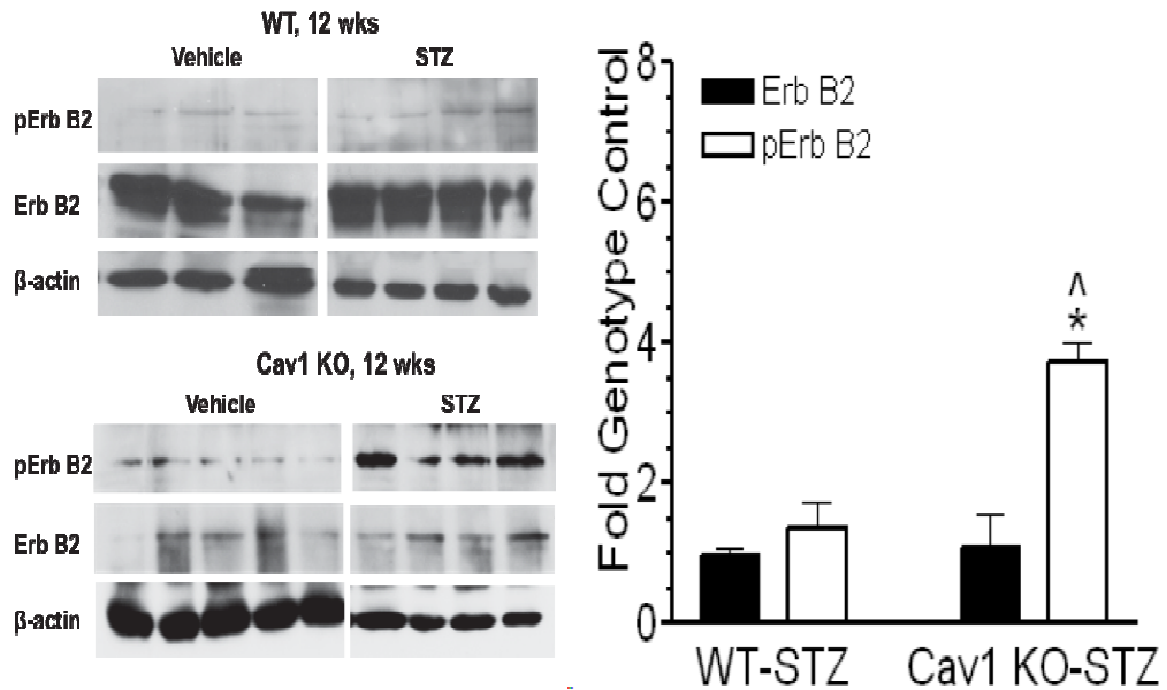


Figure 3.9: ErbB2 activation after 12 weeks of diabetes. Diabetic Cav-1^{-/-} mice demonstrate a significant increase in ErbB2 activation, evidenced by an increase in the presence of pErbB2. Levels of pErbB2 and ErbB2 are normalized to actin levels. * = $p < 0.05$ for diabetic vs. non-diabetic. [^] = $p < 0.05$ for Cav-1^{-/-} vs. wild-type

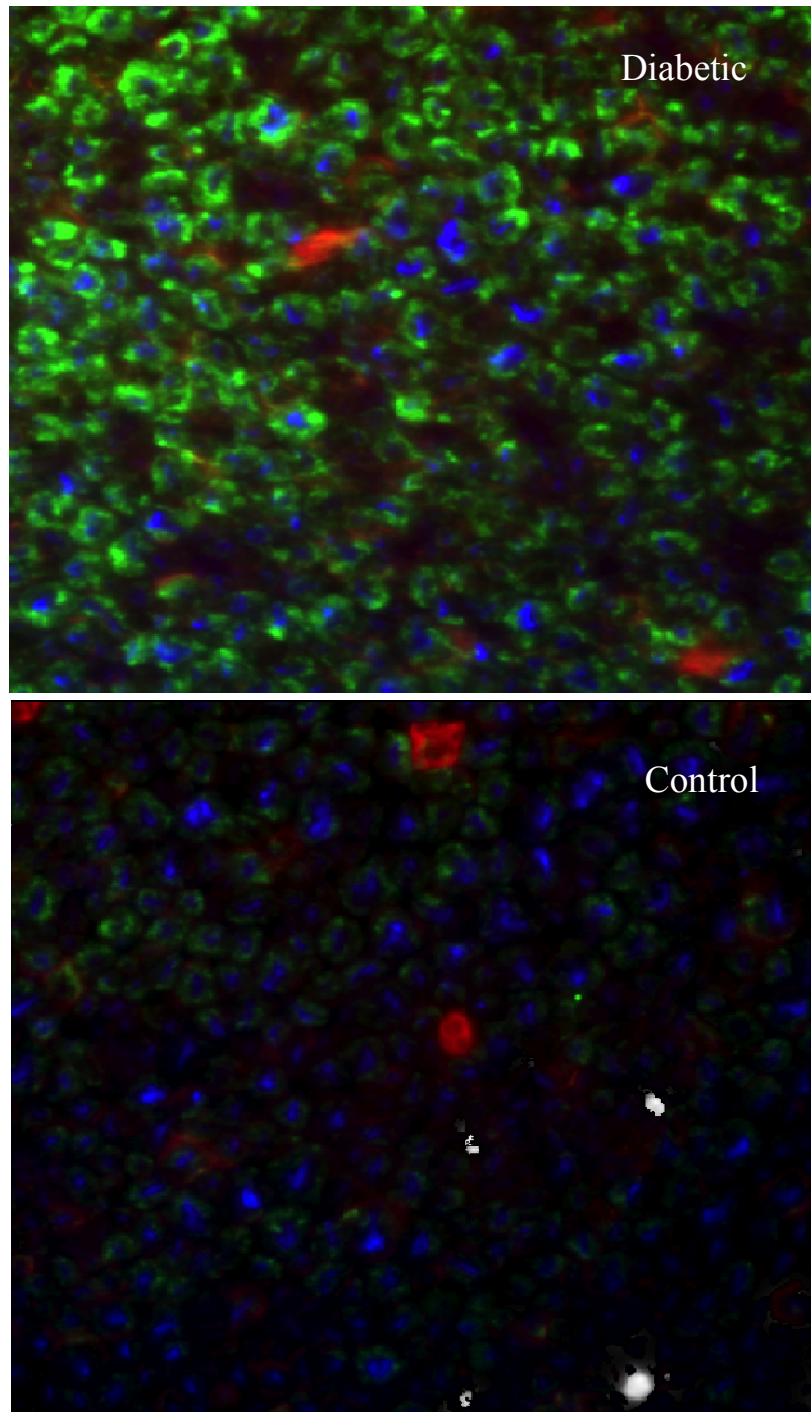


Figure 3.10: Immunofluorescence analysis of ErbB2 activation in sciatic nerve cross sections after six weeks of diabetes in wild-type mice. The presence of pErbB2 (green) was significantly greater in diabetic mice than in non-diabetic mice. Localization of pErbB2 was predominantly in the Schwann cells since the pErbB2 signal surrounded the axonal marker neurofilament – H (blue).

3.2 Hyperglycemia and ErbB2 Activation

In order to address whether the increase in ErbB2 activation observed in diabetic mice contributed to the onset and development of nerve dysfunction, we induced diabetes in Cav-1^{-/-} and wild-type mice and evaluated nerve function during and after inhibiting ErbB2 activation. We used two structurally diverse antagonists of the epidermal growth factor receptor (EGFR) family. PKI 166 is an inhibitor of the EGFR family of receptors and has been employed to demonstrate that ErbB2 activation contributes to demyelination (Guertin, Zhang et al. 2005; Tapinos, Ohnishi et al. 2006). The clinically approved EGFR family inhibitor erlotinib has also been shown to inhibit ErbB2 activity (Hernan, Fasheh et al. 2003). We observed an increase in ErbB2 activation that correlated with nerve dysfunction, therefore similar outcomes with both of these inhibitors would indicate that ErbB2 activation is contributing to the development of diabetic peripheral neuropathy.

Since we observed a significant decline in nerve function after short durations of diabetes, we sought to determine what affect inhibition of ErbB2 would have at early time periods. Cav-1^{-/-} and wild-type were made diabetic for a period of three weeks and then subgroups were treated with 25 mg/kg of PKI 166 twice a week for an additional three weeks. This dose was chosen because it had shown efficacy in inhibiting ErbB2 activation in a previous study (Guertin, Zhang et al. 2005). As had been seen before, diabetic Cav-1^{-/-} mice demonstrated a significantly more severe deficit in MNCV than diabetic wild-type mice (Figure 3.11). After three weeks of treatment with PKI 166, we observed a significant recovery of MNCV in both diabetic Cav-1^{-/-} and wild-type mice. Cav-1^{-/-} and wild-type mice both regained normal motor nerve conduction velocities, but the affect of PKI 166 on nerve function in Cav-1^{-/-} mice is more significant because their deficit was 20% greater than the deficit seen in wild-type mice. It is important to note that PKI 166 treatment alone did not

have an affect on MNCV, indicating that basal levels of ErbB2 activity do not influence motor nerve conduction velocity.

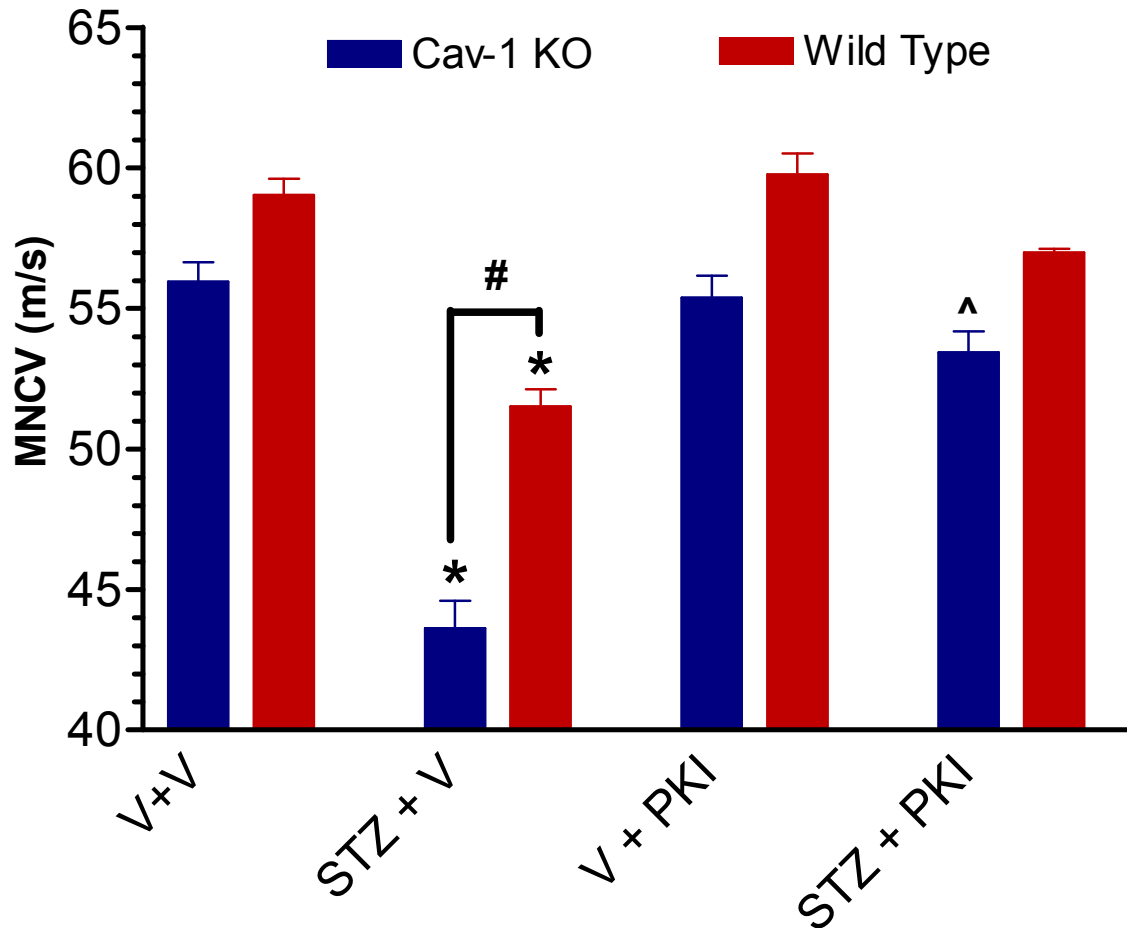


Figure 3.11: MNCV in diabetic and non-diabetic Cav-1^{-/-} and wild-type mice after treatment with the ErbB2 inhibitor PKI 166. Diabetes was induced with STZ and left untreated for three weeks. Mice were then treated biweekly with 25 mg/kg for three weeks. Untreated diabetic mice demonstrated significant MNCV impairment that was completely corrected by treatment with PKI 166. * = $p < 0.05$ for STZ + V vs. V + V. # = $p < 0.05$ for Cav-1^{-/-} STZ + V vs. wild-type STZ + V. ^ = $p < 0.05$ for STZ + PKI vs. STZ + V.

Because of the multifactoral nature of the etiology of diabetic peripheral neuropathy we were surprised the degree of improvement in MNCV conferred by PKI 166. In order to

identify the role of ErbB2 activation in nerve dysfunction over a longer period of hyperglycemia, we treated Cav-1^{-/-} and wild-type mice that were diabetic for 12 weeks with the ErbB2 antagonist erlotinib and evaluated nerve function. Diabetes was induced in Cav-1^{-/-} and wild-type mice which were untreated for 12 weeks. After 12 weeks of diabetes, mice were treated biweekly with 25 mg/kg of erlotinib. Rather than relying on NCV alone, we also assessed thermal and mechanical sensitivity before and during erlotinib treatment.

Figure 3.12 shows the response of erlotinib treated and vehicle treated Cav-1^{-/-} and wild-type

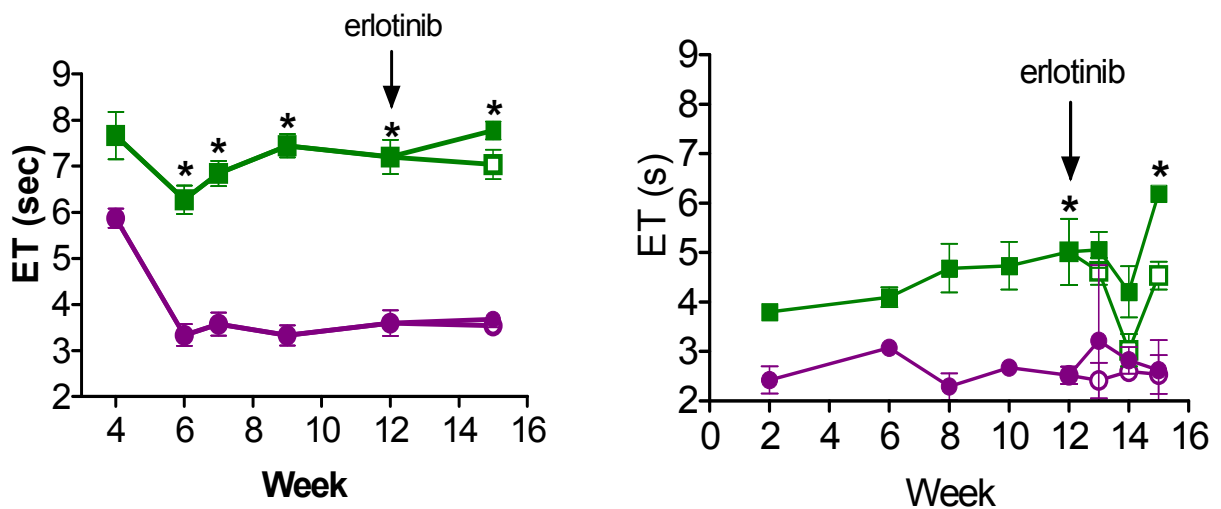


Figure 3.12: Thermal sensitivity in erlotinib treated diabetic (green squares) and non-diabetic (purple circles) Cav-1^{-/-} (left) and wild-type (right) mice. Induction of diabetes resulted in the development of thermal insensitivity. Treating with erlotinib (open squares and circles) had no affect on improving thermal insensitivity. * = p < 0.05 for diabetic vs non-diabetic.

mice. As we had seen before, diabetic Cav-1^{-/-} mice developed about a 25% reduction in thermal sensitivity. Wild-type mice also developed a significant reduction in thermal sensitivity. However, three weeks of erlotinib treatment did not improve thermal sensitivity. Three weeks of biweekly erlotinib treatment did have positive effects on mechanical sensitivity though. Similar to our initial study looking at the development of diabetic peripheral neuropathy, we again observed a significant, severe decline in mechanical

sensitivity in diabetic Cav-1^{-/-} mice and eventually in diabetic wild-type mice (Figure 3.13).

After 12 weeks of diabetes, mice became insensitive to mechanical stimulation and 10 – 15% more force was required for paw withdrawal in these mice as opposed to non-diabetic mice.

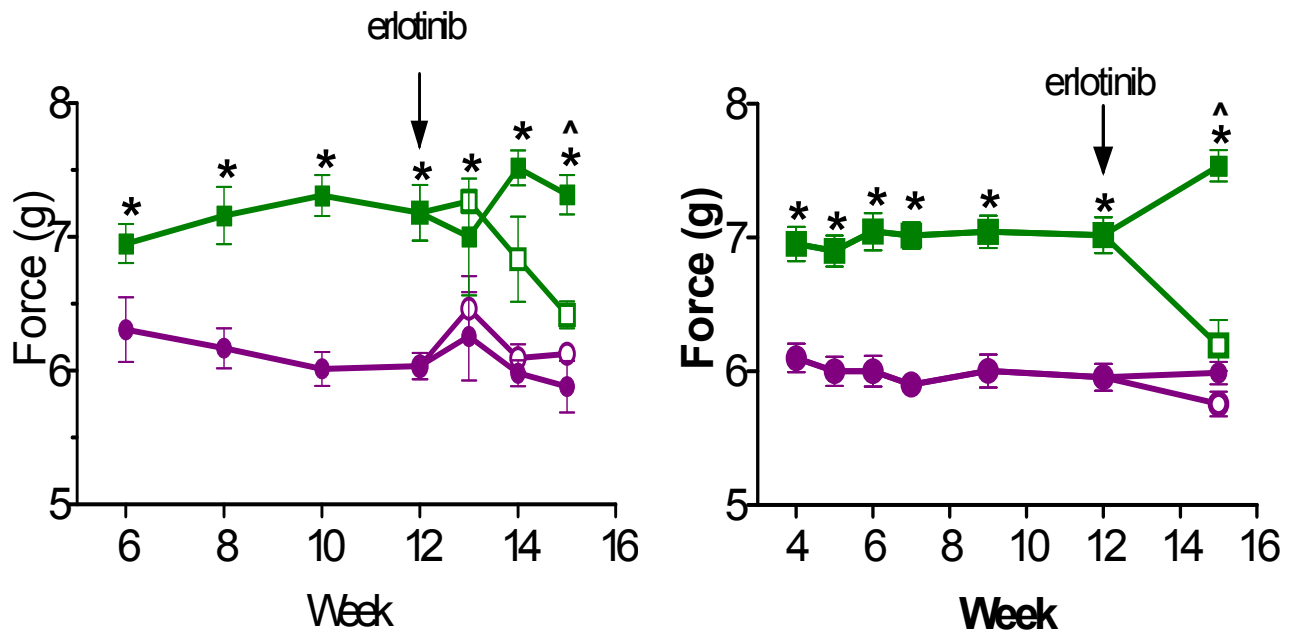


Figure 3.13: Mechanical sensitivity in erlotinib treated diabetic (green squares) and non-diabetic (purple circles) Cav-1^{-/-} (left) and wild-type (right) mice. Induction of diabetes resulted in the development of mechanical insensitivity. Treating with erlotinib (open squares and circles) allowed for the recovery from deficits in mechanical sensitivity. * = p < 0.05 for diabetic vs non-diabetic mice. ^ = p < 0.05 for erlotinib treated vs. vehicle treated mice.

Inhibiting ErbB2 activation with biweekly treatment of erlotinib resulted in a near complete recovery from deficits in mechanical sensitivity in diabetic Cav-1^{-/-} and wild-type mice.

After 12 weeks of STZ induced diabetes Cav-1^{-/-} and wild-type mice developed similar MNCV deficits to what we had seen previously (Figure 3.14). Motor nerve conduction velocity was about 20% lower in diabetic Cav-1^{-/-} mice than in non-diabetic Cav-1^{-/-} mice.

Likewise, diabetic wild-type mice also demonstrated a nearly 20% drop in MNCV compared to non-diabetic mice. Three weeks of treatment with erlotinib led to a partial recovery of

these deficits in both genotypes. This correction in MNCV suggests that after longer periods of hyperglycemia, neuropathy can only partially be explained by the over-activation of

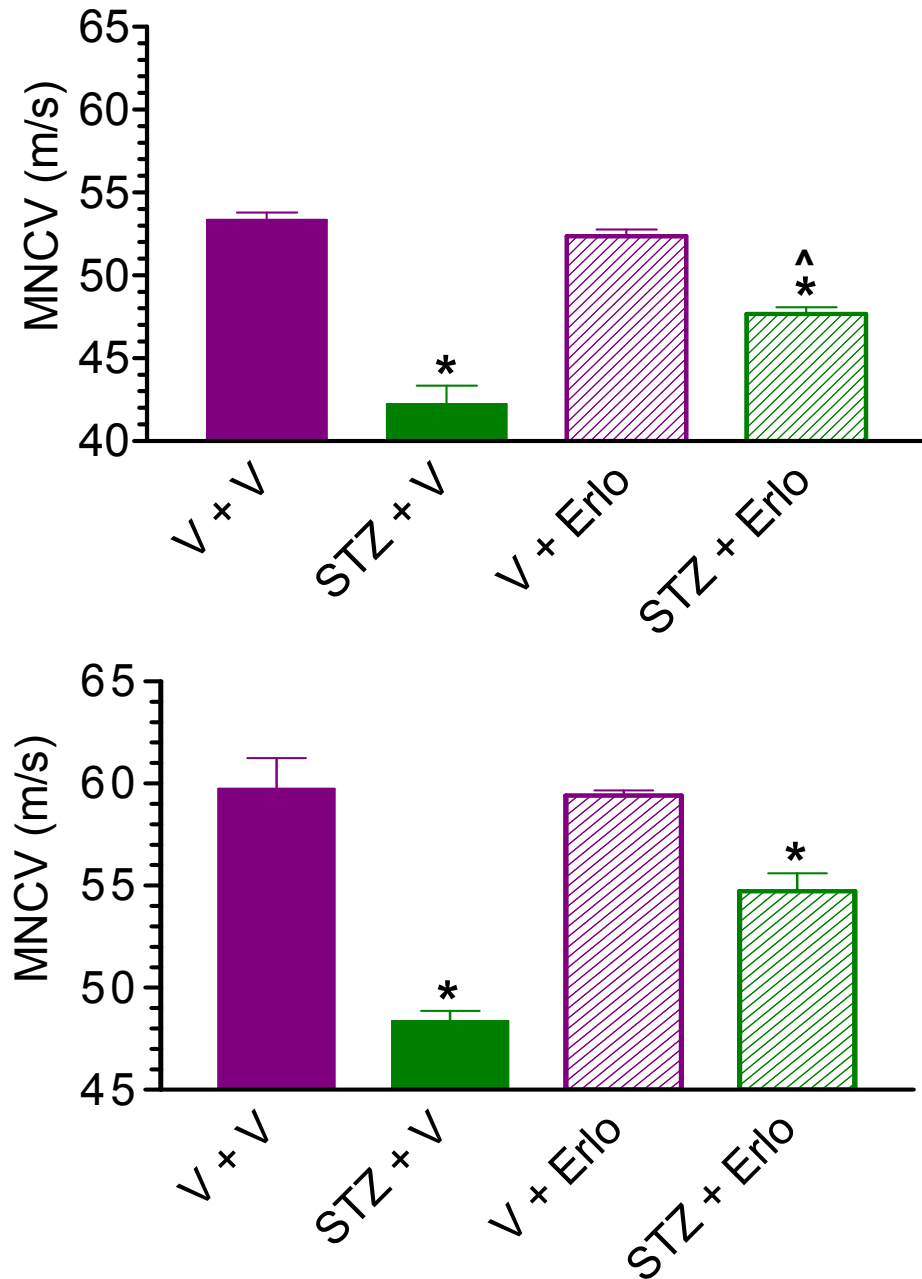


Figure 3.14: MNCV in erlotinib and vehicle treated Cav-1^{-/-} (top) and wild-type mice (bottom). Diabetic mice developed a substantial MNCV deficit after 12 weeks of diabetes. Treatment with 25 mg/kg erlotinib led to a partial correction of this deficit in both genotypes. * = $p < 0.05$ for untreated STZ vs. untreated V. ** = $p < 0.05$ for STZ + V for V + V. # = $p < 0.05$ for STZ + Erlo vs. STZ + V. ^ = $p < 0.05$ for STZ + Erlo vs. V + Erlo

ErbB2. As diabetes progresses, changes in the metabolic pathways described earlier begin eliciting their injurious effects on nerves. Consequently, the over-activation of ErbB2 is not the only factor leading to nerve dysfunction. Therefore, inhibiting ErbB2 activation results in a partial recovery of behavioral and electrophysiological deficits.

In order to identify the role of ErbB2 activation in the development and maintenance of diabetic peripheral neuropathy, we performed a similar experiment as the previous one, with the main difference being the duration of diabetes. Instead of mice being diabetic for 12 weeks and treated with an ErbB2 inhibitor for 3 weeks, wild-type mice were made diabetic and left untreated for 20 weeks and then treated with an ErbB2 inhibitor for an additional 6 weeks. Similar to previous results, STZ treatment resulted in about a three- to fourfold increase in fasting plasma glucose, more than doubling of glycated hemoglobin, and a reduction in weight gain (Table 3.2).

Table 3.2: Weight, fasting plasma glucose (BG), glycated hemoglobin (Hb A1C), and N for diabetic and non-diabetic wild-type mice. Mean \pm SD.

Duration of Diabetes (weeks)	STZ				Vehicle			
	Weight	BG (mg/dL)	Hb A1C	N	Weight	BG (mg/dL)	Hb A1C	N
0	21.41 \pm 2.25	347.50 \pm 34.00			16.07 \pm 0.66	89.00 \pm 12.49		
2	20.15 \pm 1.43				21.40 \pm 0.58			
4	21.49 \pm 1.54				21.45 \pm 0.75			
6	22.66 \pm 1.11				21.83 \pm 1.16			
8	23.03 \pm 1.77				22.56 \pm 0.88			
10	23.63 \pm 1.48	422.50 \pm 83.20			23.51 \pm 2.07	116.30 \pm 14.47		
12	23.76 \pm 1.49				23.97 \pm 1.69			
14	25.16 \pm 2.04				24.17 \pm 1.44			
16	26.05 \pm 1.81				24.55 \pm 1.36			
18	25.17 \pm 1.39				24.83 \pm 2.21			
20	21.91 \pm 2.45	263.25 \pm 26.45	9.50 \pm 1.00	4	24.20 \pm 2.00	117.70 \pm 13.32		3

Duration of Diabetes (weeks)	STZ + Erlotinib				Vehicle + Erlotinib			
	Weight	BG (mg/dL)	Hb A1C	N	Weight	BG (mg/dL)	Hb A1C	N
0	23.66 \pm 2.55	361.75 \pm 28.21			22.81 \pm 2.27	116.60 \pm 10.92		
2	21.15 \pm 1.50				24.78 \pm 1.12			

4	21.79 ± 1.86					25.98 ± 1.79			
6	22.98 ± 1.71					27.44 ± 2.20			
8	23.39 ± 1.40					29.15 ± 2.26			
10	23.35 ± 1.90	486.38 ± 28.54				30.58 ± 2.53	138.40 ± 13.74		
12	24.12 ± 1.82					31.68 ± 2.68			
14	25.02 ± 1.91					31.93 ± 3.10			
16	26.25 ± 1.69					32.61 ± 2.97			
18	25.27 ± 2.01					32.83 ± 3.14			
20	22.76 ± 2.30	415.25 ± 70.00				31.99 ± 2.85	85.60 ± 9.48		
22	24.88 ± 2.11					33.84 ± 2.41			
24	25.24 ± 2.15					33.88 ± 2.82			
26	25.13 ± 2.58	577.63 ± 79.94	10.82 ± 0.86	8		34.40 ± 2.46	130.00 ± 19.25	4.40 ± 0.0	5

Duration of Diabetes (weeks)	STZ - Erlotinib				Vehicle - Erlotinib			
	Weight	BG (mg/dL)	Hb A1C	N	Weight	BG (mg/dL)	Hb A1C	N
0	23.31 ± 2.91	384.80 ± 46.41			22.21 ± 3.40	106.00 ± 8.45		
2	20.52 ± 1.87				23.24 ± 2.80			
4	20.93 ± 1.80				23.83 ± 3.65			
6	22.31 ± 1.89				24.83 ± 3.09			
8	22.66 ± 1.74				25.76 ± 4.34			
10	22.54 ± 2.04	428.13 ± 112.30			26.82 ± 4.68	137.50 ± 14.29		
12	22.78 ± 2.16				27.96 ± 4.27			
14	24.94 ± 2.08				28.70 ± 4.74			
16	26.15 ± 1.38				29.58 ± 5.25			
18	23.80 ± 2.60				29.33 ± 5.60			
20	22.39 ± 2.14	434.00 ± 70.80			29.43 ± 4.09	116.50 ± 15.26		
22	23.95 ± 2.38				29.95 ± 5.89			
24	24.06 ± 2.36				30.14 ± 5.91			
26	23.63 ± 2.54	528.90 ± 75.95	11.59 ± 1.01	9	30.91 ± 5.54	146.00 ± 9.45	4.47 ± 0.15	4

After confirmation of hyperglycemic status, we evaluated nerve function via the Von Frey and Hargreaves methods every week for twenty weeks. After twenty weeks, several diabetic and non-diabetic mice were sacrificed to obtain pre-treatment NCV measures and tissues for morphological analysis. Remaining mice continued to be subjected to behavioral assessments every week as well as bi-weekly treatment with erlotinib. After four weeks of diabetes, diabetic mice demonstrated a significant reduction in thermal sensitivity (Figure 3.15). Over the course of the 20 weeks before treatment, diabetic mice on average required twice as long to withdraw their paws than did non-diabetic mice. Although treatment with

erlotinib prevented a worsening of thermal insensitivity, diabetic mice did not recover from the thermal insensitivity that had developed. It is possible that longer periods of erlotinib treatment could lead to a significant recovery of sensation, but this has yet to be determined.

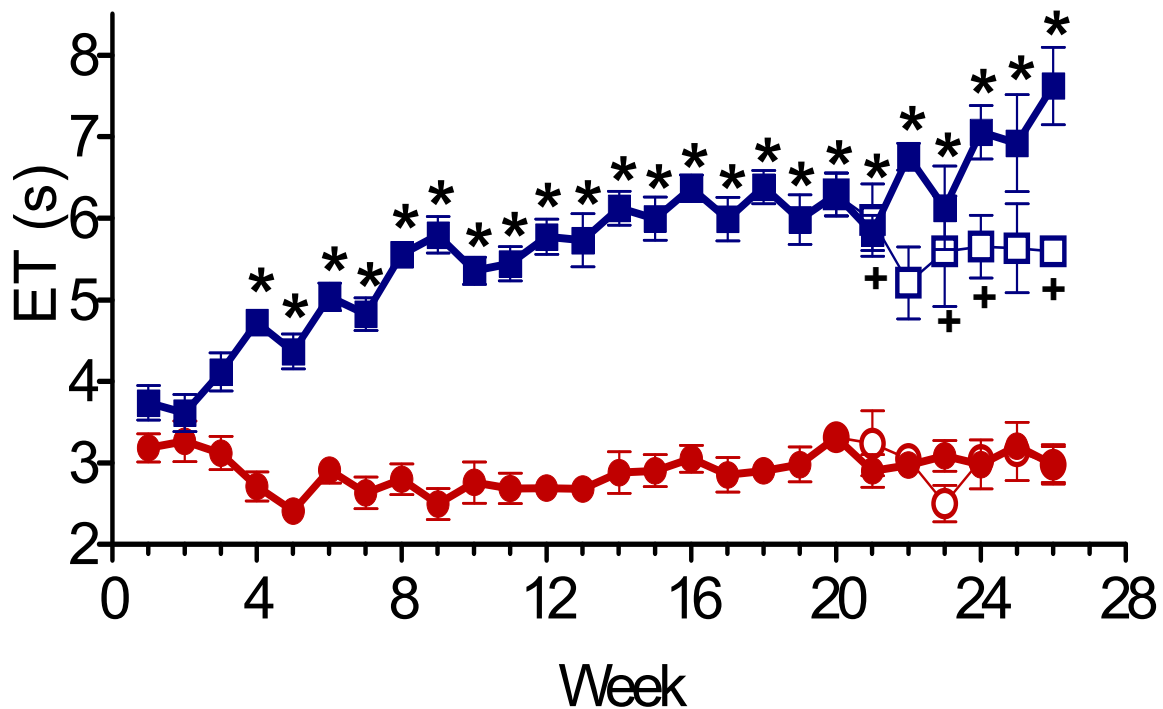


Figure 3.15: Thermal sensitivity in diabetic (blue squares) and non-diabetic (red circles) mice before and during biweekly treatment with 25 mg/kg erlotinib (open symbols). Diabetic mice demonstrated a significant decline in thermal sensitivity after four weeks of diabetes. Treatment with erlotinib led to an insignificant recovery of thermal sensitivity. * = $p < 0.05$ for diabetic vs. non-diabetic. + = $p < 0.05$ for STZ + Erlo vs. V + Erlo.

Results from the assessment of mechanical sensitivity were similar to those obtained during our first study employing erlotinib, where diabetic mice developed a sustained 20% decrease in sensitivity which was reversed by treatment with erlotinib. In this longer-term study, diabetic mice developed a significant mechanical insensitivity prior to biweekly treatment with erlotinib (Figure 3.16). After two weeks of erlotinib treatment, mechanical sensitivity in diabetic mice was significantly higher than in untreated diabetic mice and similar to non-diabetic mice. It is important to note that the insignificant recovery in thermal sensitivity and

the full recovery in mechanical insensitivity cannot be attributed to basal ErbB2 activity because erlotinib treatment had no affect on non-diabetic mice.

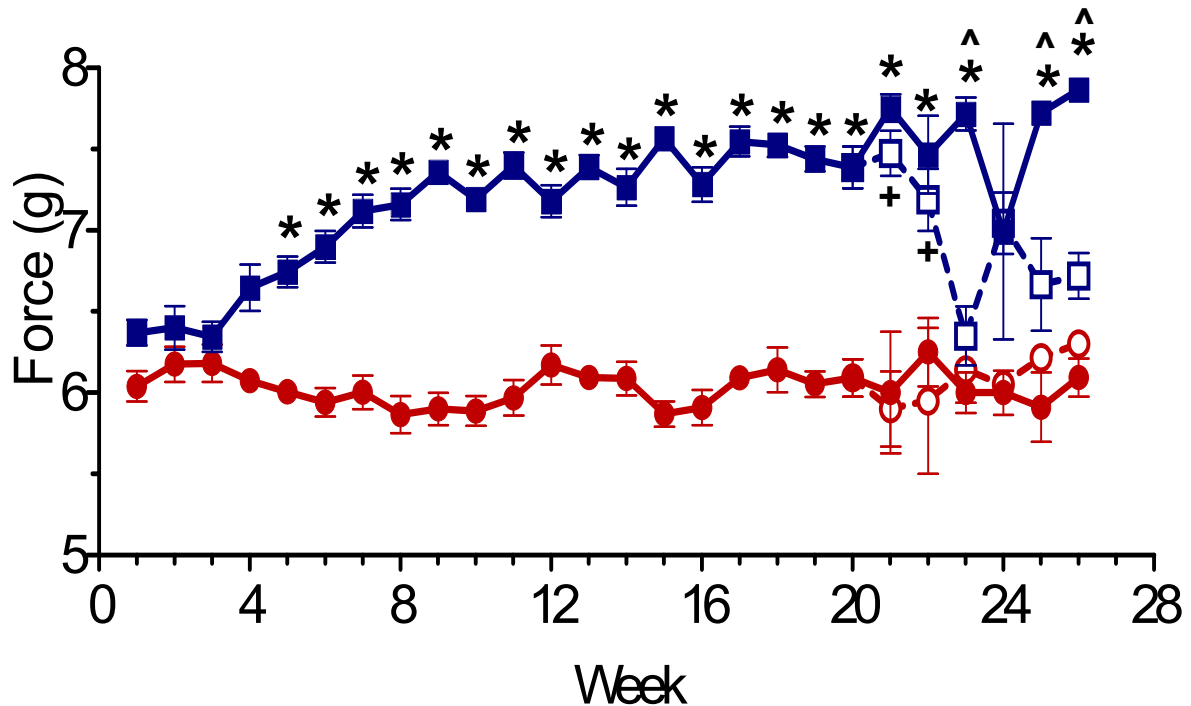


Figure 3.16: Mechanical sensitivity in diabetic (blue squares) and non-diabetic (red circles) mice before and during biweekly treatment with 25 mg/kg erlotinib (open symbols). Diabetic mice demonstrated a significant decline in mechanical sensitivity after five weeks of diabetes. Two weeks of erlotinib treatment led to a significant recovery of mechanical sensitivity. * = $p < 0.05$ for diabetic vs. non-diabetic. ^ = $p < 0.05$ for STZ + Erlo vs. STZ + V. + = $p < 0.05$ for STZ + Erlo vs. V + Erlo.

In order to determine whether or not inhibition of ErbB2 after a long period of diabetes would reverse MNCV deficits in a similar manner as it did after 12 weeks of diabetes, we measured NCV before treatment with erlotinib and then after six weeks of erlotinib treatment. Since previous results indicated that diabetic mice demonstrate a significant loss of MNCV we expected to observe similar or more severe deficits after 20 weeks of hyperglycemia. Indeed, diabetic wild-type mice developed about a 20% deficit in MNCV as we had seen before (Figure 3.17). After six weeks of erlotinib treatment, diabetic

wild-type mice demonstrated a partial reversal of the MNCV deficit. Although conduction

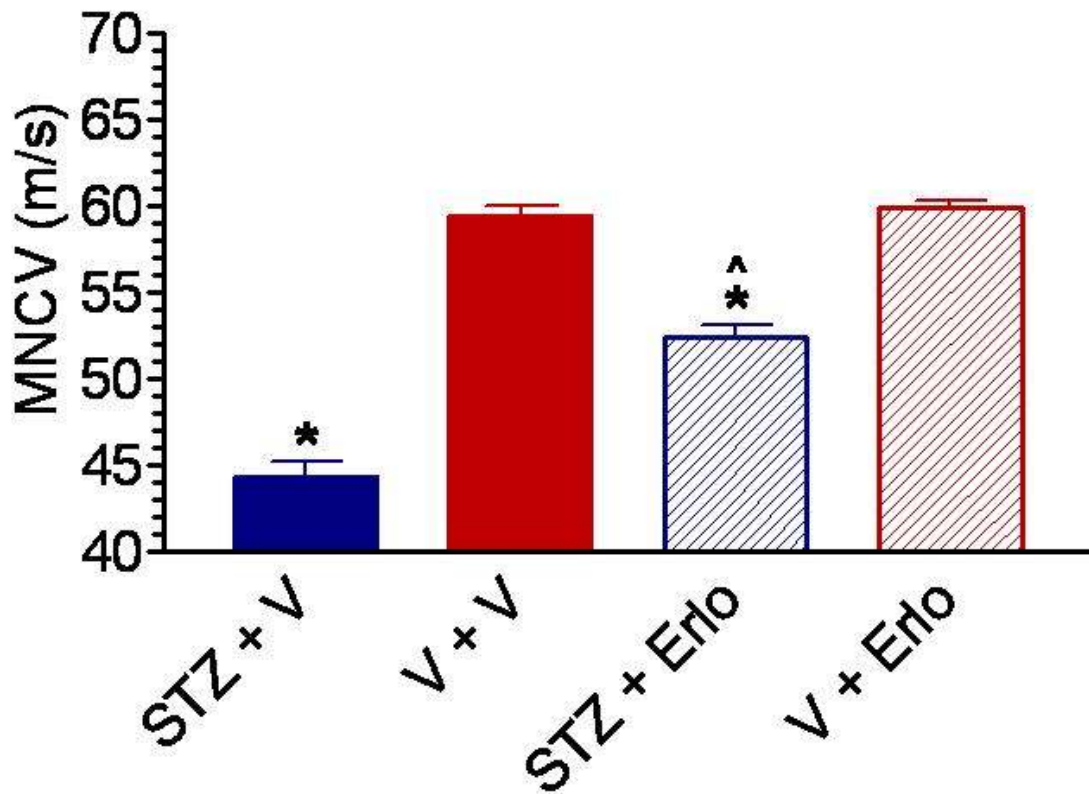


Figure 3.17: MNCV in wild-type mice before and after treatment with erlotinib. Diabetic mice developed a significant deficit in MNCV after 20 weeks of STZ induced diabetes (STZ and V). Biweekly treatment with erlotinib resulted in a significant, partial recovery from this deficit (STZ + V, V + V, STZ + Erlo, V + Erlo). Erlotinib treated diabetic mice had significantly improved MNCV but were still significantly deficient compared to non-diabetic mice. * = $p < 0.05$ for STZ vs. V. ^ = $p < 0.05$ for STZ + Erlo vs. V + Erlo.

was significantly greater in erlotinib treated mice, it remained significantly slower than non-diabetic mice. It is important to note that basal levels of ErbB2 still did not have an impact MNCV or SNCV since these values were similar in non-treated controls and non-diabetic treated mice. Additionally, we observed for the first time a significant reduction in SNCV after 20 weeks of diabetes (Figure 3.18). Diabetic mice developed an SNCV deficit of nearly 20%. Treatment with erlotinib though did not have an affect on SNCV as nerve conduction

velocity was similar in erlotinib treated and untreated diabetic mice.

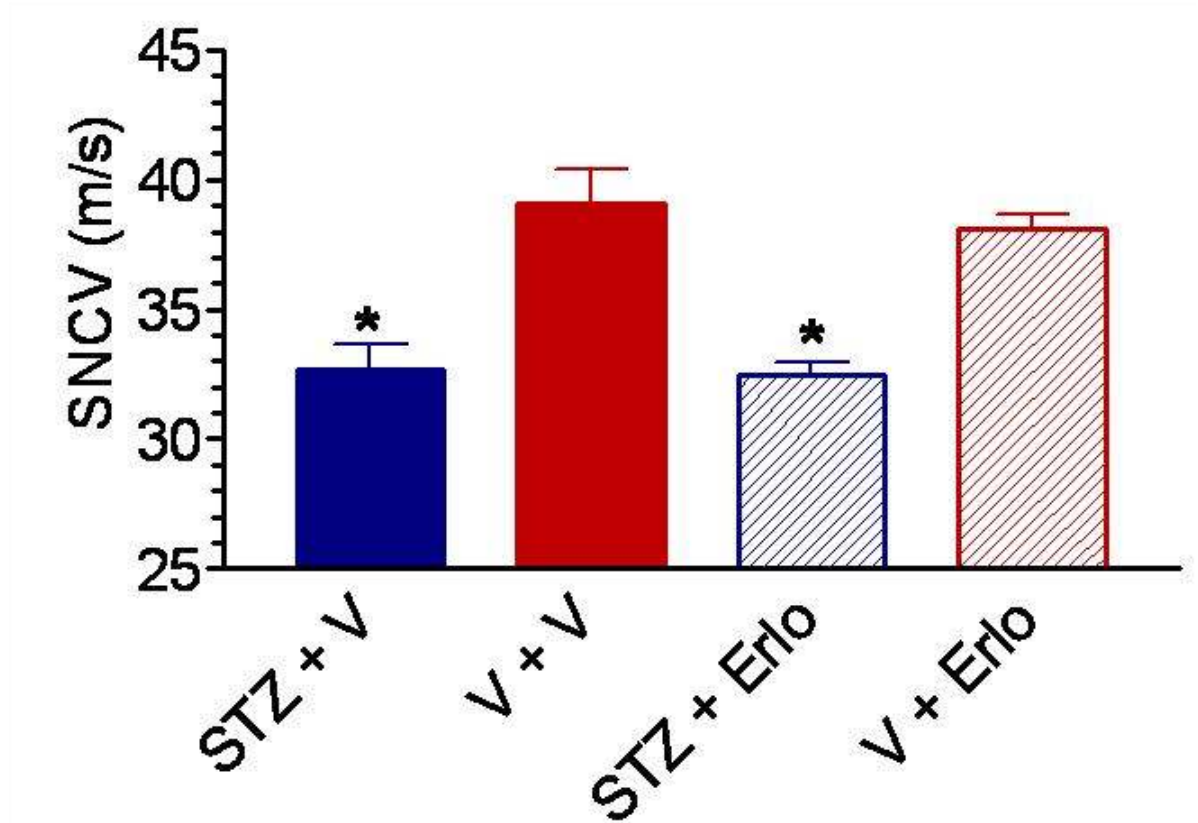


Figure 3.18: SNCV in wild-type mice before and after treatment with erlotinib. Diabetic mice developed a significant deficit in SNCV after 20 weeks of STZ induced diabetes (STZ and V). Biweekly treatment with erlotinib did not have an affect on SNCV (STZ + V, V + V, STZ + Erlo, V + Erlo). * = $p < 0.05$ for STZ vs. V

Mechanical sensitivity and motor nerve conduction velocity are functions of myelinated nerves and the evidence we have gathered suggests that these fibers are more susceptible to ErbB2 mediated damage than unmyelinated fibers. To address whether or not hyperglycemia promotes demyelination and if inhibition of ErbB2 could prevent such demyelination, we calculated the g-ratio in diabetic and non-diabetic wild-type mice. Sciatic nerves from the mice used for behavioral and electrophysiologic assessments were removed and fixed immediately following euthanasia. The g-ratio was then calculated for mice before and after treatment with erlotinib. As can be seen in Figure 3.19, STZ induced

hyperglycemia had no affect on the g-ratio after 20 or 26 weeks. Furthermore, treatment with erlotinib had no impact on g-ratio as well.

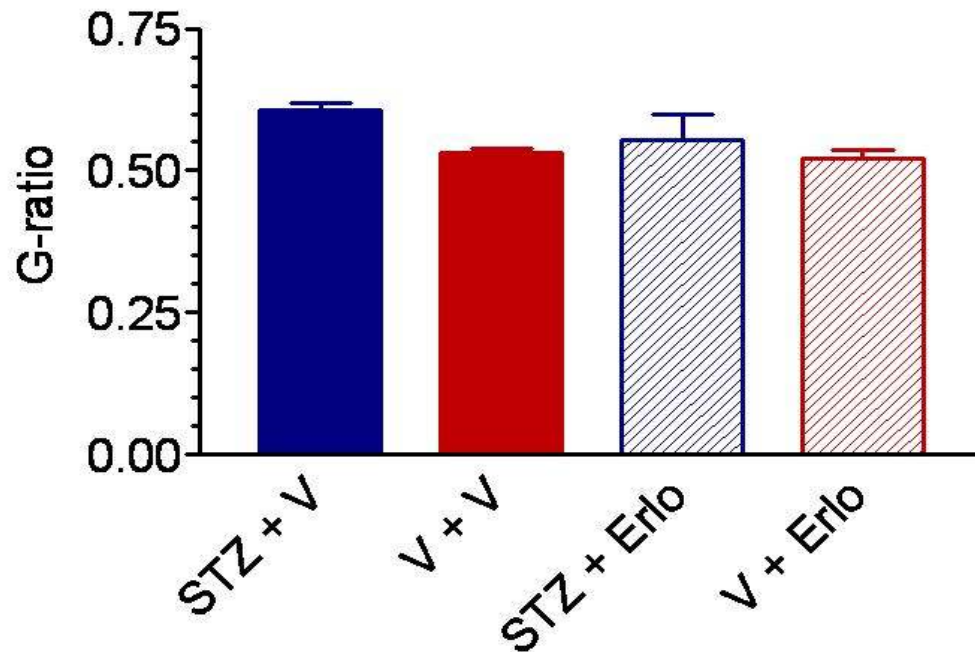
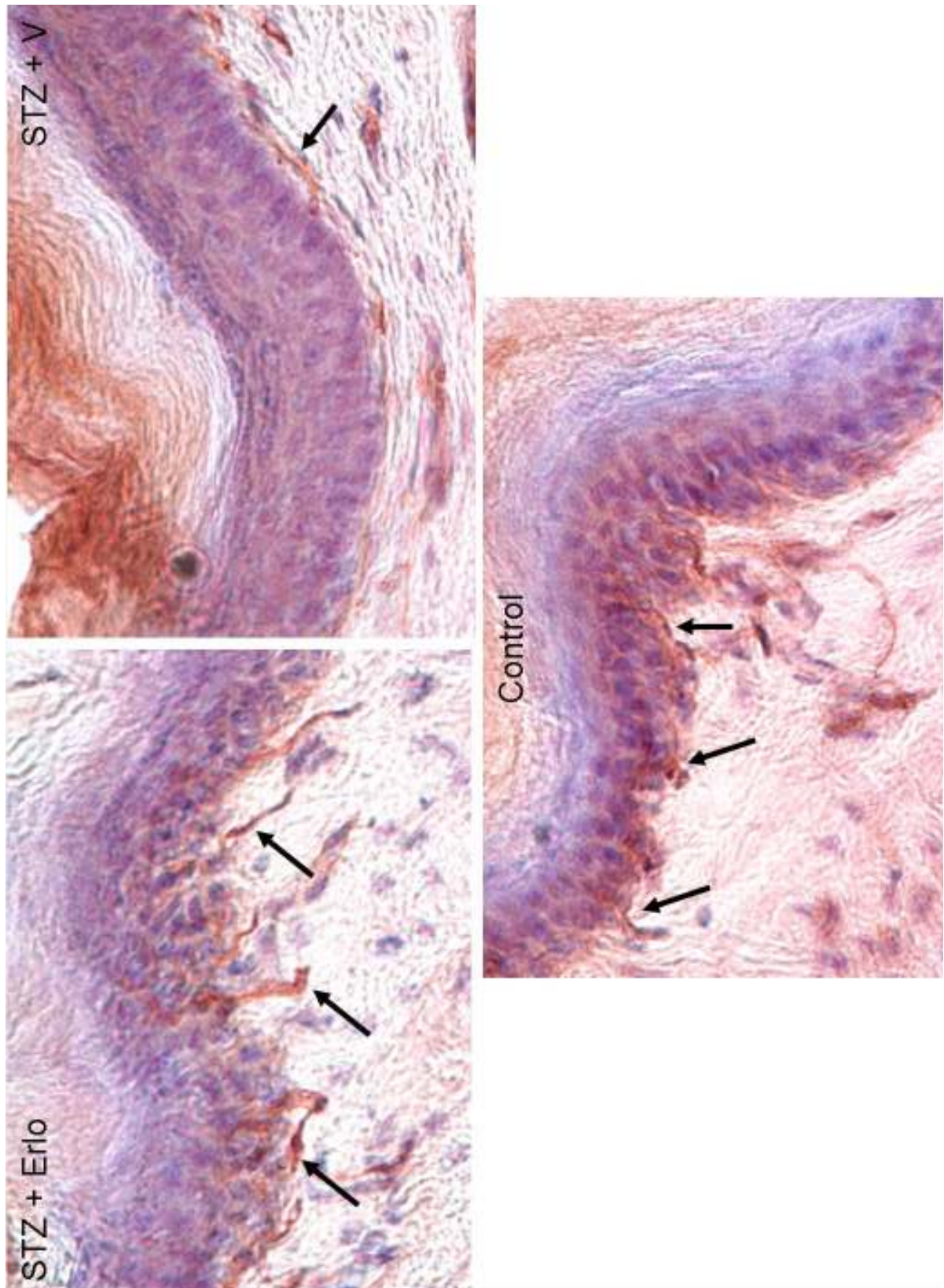


Figure 3.19: g-ratio quantification in diabetic and non-diabetic wild-type mice before and after erlotinib treatment. STZ induced diabetes did not have an affect on the g-ratio in wild-type mice after 20 weeks (STZ and V) and 26 weeks (STZ + V, V + V, STZ + Erlo, V + Erlo).

In order to determine if deficits in mechanical and thermal sensitivity could be attributed to a loss of innervation of the plantar surface of the hind paw, we quantified IENFD in diabetic and non-diabetic mice before and after being subjected to biweekly treatment with 25 mg/kg erlotinib. Recall that after 12 weeks of STZ induced diabetes we did not observe any changes in epidermal innervation in diabetic mice (Figure 3.5). However, after 20 weeks of diabetes we observed an insignificant decline in innervation that became significantly worse after 26 weeks (Figure 3.20). After 26 weeks of hyperglycemia, untreated diabetic mice demonstrated a greater than 20% decrease in the number of nerve

fibers crossing the dermal – epidermal junction. Surprisingly, six weeks of erlotinib treatment resulted in a complete correction of nerve fiber loss. These results suggest that the over-activation of ErbB2 for an extended period of time mediates the loss of epidermal nerve fibers and that at least a portion of the behavioral deficits observed after long periods of diabetes can be attributed to the loss of epidermal fibers. However, our results also indicate that this is not the case over the entire time course of STZ induced diabetes, since no differences in innervation were observed after 12 weeks of diabetes. Only after an extended period of hyperglycemia do epidermal fibers disappear.

A



B

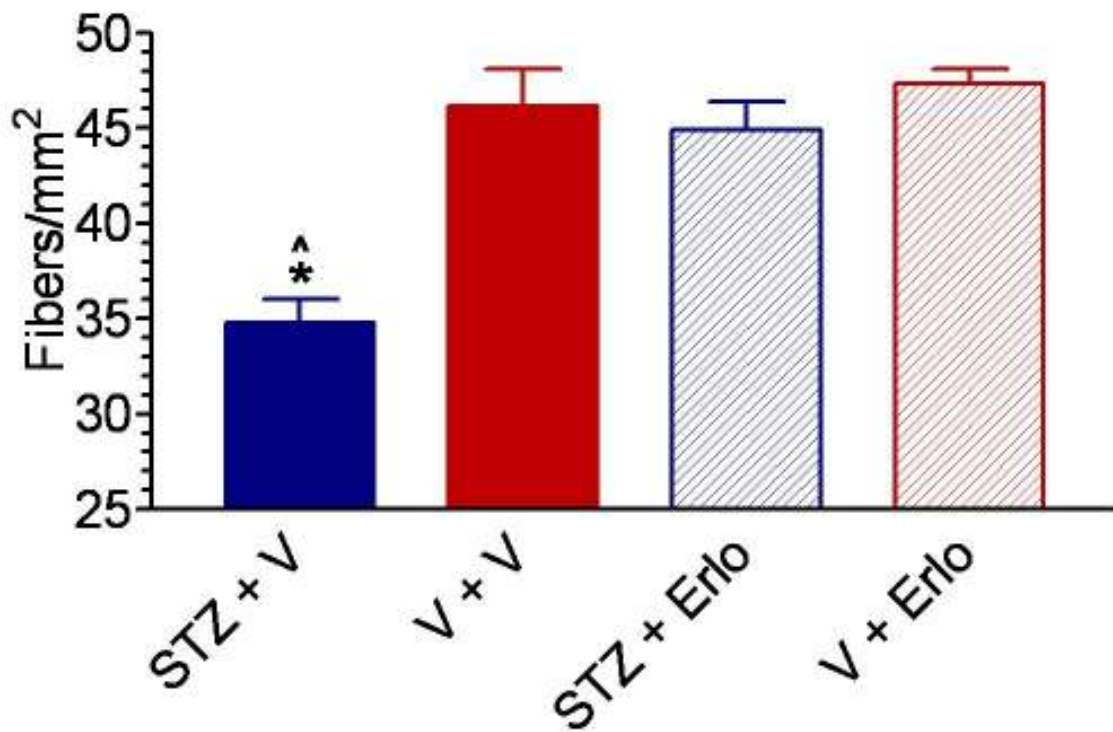


Figure 3.20: Epidermal innervation in diabetic and non-diabetic mice before and after treatment with erlotinib: A) Representative images from erlotinib treated and untreated mice. Epidermal cells are stained purple and nerve fibers are stained red (arrows). B) Untreated diabetic mice demonstrated a significant loss of epidermal fibers while erlotinib treated mice demonstrated a recovery from this loss. * = $p < 0.05$ for STZ vs. V. ^ = $p < 0.05$ for STZ + V vs. STZ + Erlo

3.3 ErbB2 Activation in the Absence of Hyperglycemia

In order to establish whether or not increased ErbB2 activity in the absence of hyperglycemia is sufficient to induce a neuropathic phenotype, we developed a conditional double transgenic mouse line (P₀rtTA x caErbB2) that expresses a constitutively active form of ErbB2 in myelinating Schwann cells. Since PKI 166 and erlotinib are not ErbB2 selective antagonists and may inhibit other members of the EGFR family, using a conditional transgenic over-expressing ErbB2 would allow us to confirm that these inhibitors are eliciting their effects through the ErbB2 receptor. After confirming the presence and inducibility of the transgene (Figure 2.2), we induced expression of the constitutively active ErbB2 and evaluated nerve function in these mice. Since the constitutively active form of ErbB2 is limited to myelinated Schwann cells, we would not expect to see any changes in the function or conduction velocity in non-myelinated nerve fibers. Figure 3.21 shows that this is the case when evaluating thermal sensitivity in P₀rtTA x caErbB2 mice. P₀rtTA x caErbB2 mice treated with doxycycline demonstrated similar thermal sensitivity to mice not treated

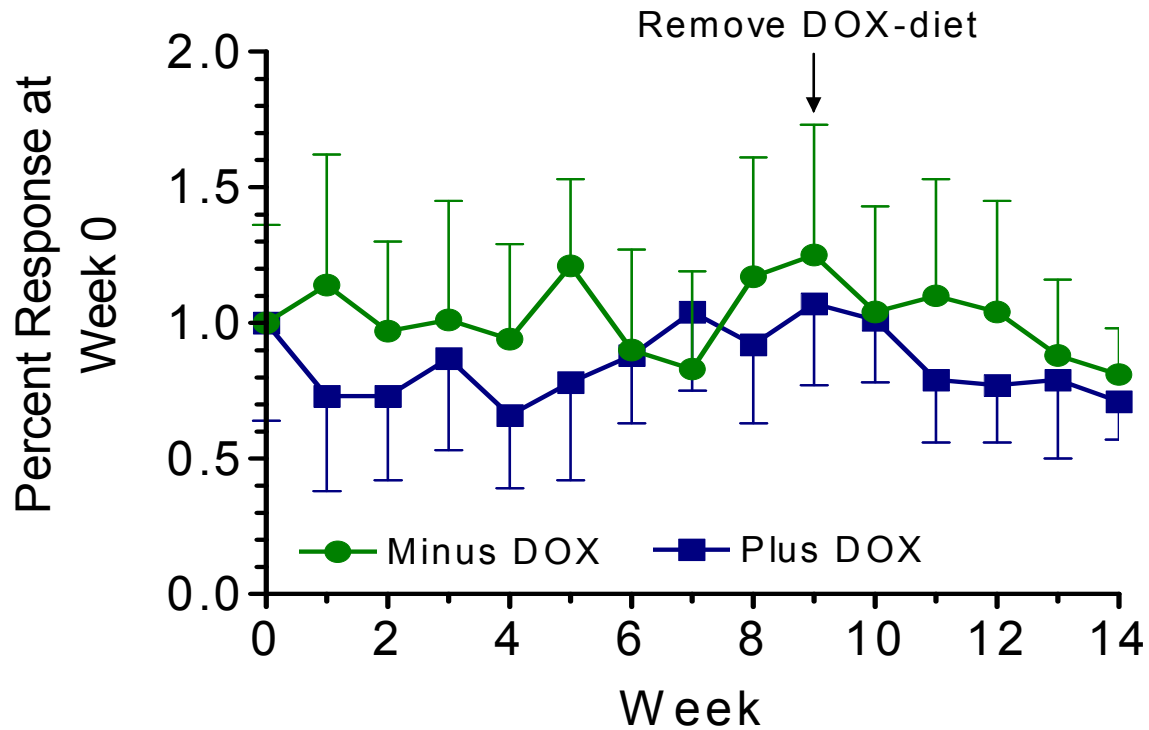


Figure 3.21: Thermal sensitivity in P_{0rtTA} x $caErbB2$ mice treated with doxycycline (Dox). Mice treated with Dox did not demonstrate deficits in thermal sensitivity.

with doxycycline for 14 weeks. Furthermore, the removal of doxycycline to stop transgene induction after nine weeks did not impact thermal sensitivity. Nine weeks of doxycycline though did adversely affect mechanical sensitivity. Mice treated with doxycycline began to demonstrate at least a 20% deficit in mechanical sensitivity after 4 weeks (Figure 3.22). The removal of doxycycline after nine weeks corrected the deficit in mechanical sensitivity. These results suggest that the over-activation of ErbB2 alone is sufficient to elicit a

neuropathic phenotype.

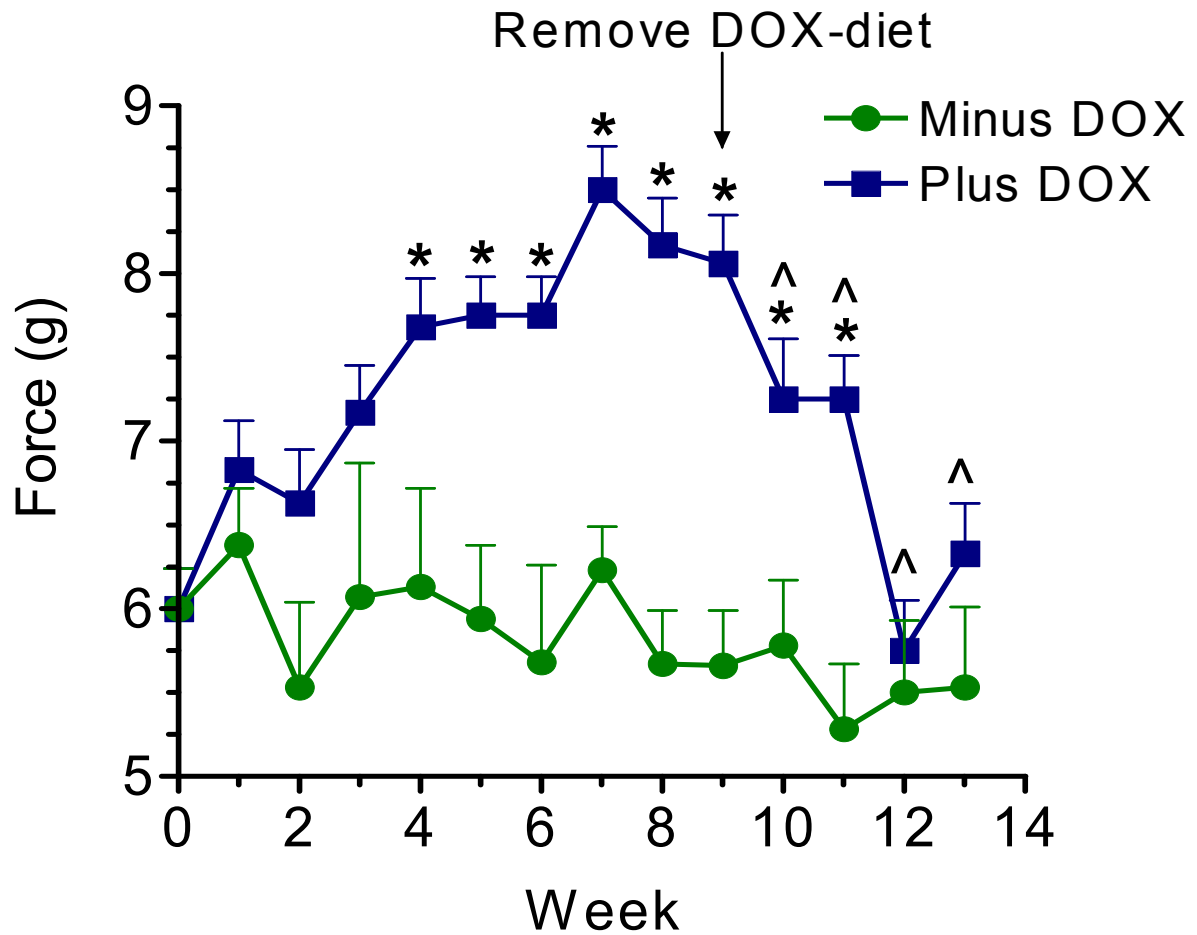


Figure 3.22: Mechanical sensitivity in P₀rtTA x caErbB2 mice treated with doxycycline (Dox). Mice treated with Dox demonstrated a significant reduction in mechanical sensitivity. Removal of Dox treatment corrected this deficit, as sensitivity became significantly higher once Dox was removed. * = $p < 0.05$ for Dox treated vs. untreated. ^ = $p < 0.05$ for response vs. Dox treatment at week 9.

To confirm that the neuropathic phenotype induced by the treatment with doxycycline was the result of ErbB2 activation, we treated another group of mice with doxycycline for four weeks and then treated biweekly with 25 mg/kg PKI 166 for three weeks. After four weeks of doxycycline treatment, mice develop about a 25% decline in MNCV (Figure 3.23). The concomitant treatment of doxycycline and PKI 166 reverses the deficit in MNCV. Mice

treated with doxycycline and PKI 166 have the same nerve conduction velocities as mice not treated with doxycycline, suggesting the deficits in mechanical sensitivity and motor nerve conduction are the result of over-activation of the ErbB2 receptor.

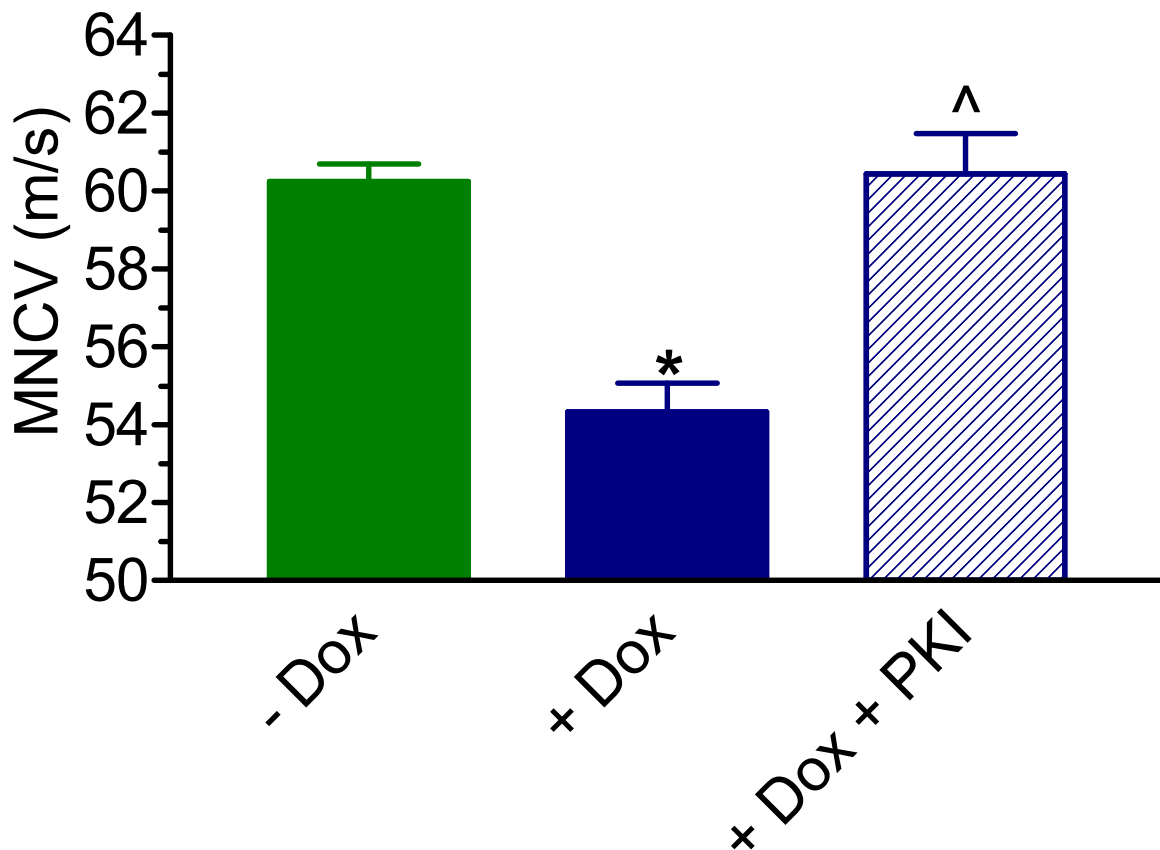


Figure 3.23: MNCV in doxycycline (DOX) treated $P_{0rtTA} \times caErbB2$ mice. Four weeks of DOX treatment produced a significant reduction in MNCV. Three weeks of treatment with the ErbB2 inhibitor PKI 166 completely restored MNCV. * = $p < 0.05$ for -Dox vs. +Dox. ^ = $p < 0.05$ for +Dox vs. +Dox + PKI 166.

Since our initial period of doxycycline treatment was short, we wanted to determine if the development of a neuropathic phenotype after longer periods of doxycycline treatment could be attributed to ErbB2 activation. Recall that when we inhibited ErbB2 activation after longer periods of diabetes, we did not observe a full restoration of deficits. These results

suggested that other factors aside from the activation of ErbB2 contribute to the progression of neuropathy after longer periods of hyperglycemia. To ensure that ErbB2 activation was the cause of the neuropathic phenotype in $P_{0rtTA} \times caErbB2$ mice, we induced expression of the constitutively active form of ErbB2 in Schwann cells for 12 weeks and then treated mice with the ErbB2 inhibitor erlotinib. Again, since our double transgenic mouse line was designed to limit the expression of the constitutively active form of ErbB2 to Schwann cells, we would not expect any changes in SNCV. Treatment with doxycycline did not result in a change, either positive or negative, in SNCV (Figure 3.24). However, after three weeks of doxycycline treatment mice develop about a 20% deficit in MNCV (Figure 3.25).

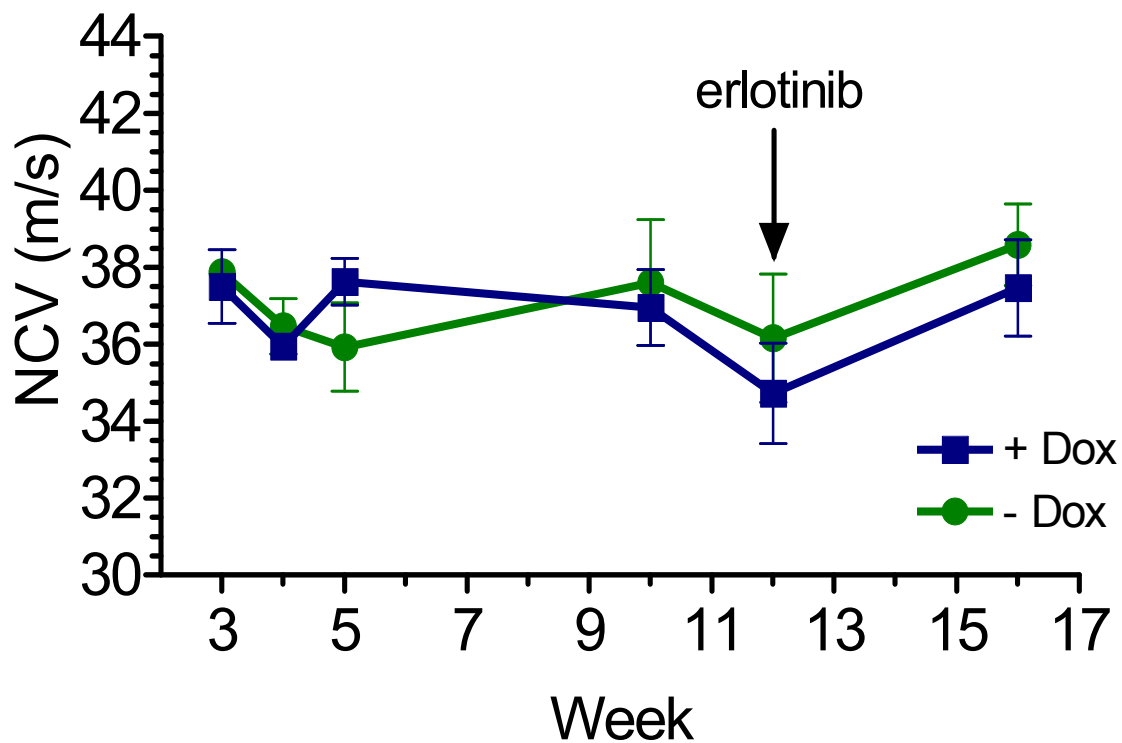


Figure 3.24: Sensory nerve conduction velocity in $P_{0rtTA} \times caErbB2$ mice treated with doxycycline (Dox). Mice treated with Dox did not demonstrate deficits in SNCV after 12 weeks and treatment with erlotinib had no affect on SNCV.

This deficit is maintained through 12 weeks of doxycycline treatment and is reversed when mice were treated with erlotinib. Biweekly treatment with 25 mg/kg of erlotinib fully

restores the observed deficits in MNCV, suggesting that the development of a neuropathic phenotype in these mice is fully attributable to an increase in the activation of ErbB2.

Additionally, these results suggest that the development of neuropathy in diabetic wild-type and Caveolin-1 mice is also partially attributable to the over-activation of ErbB2.

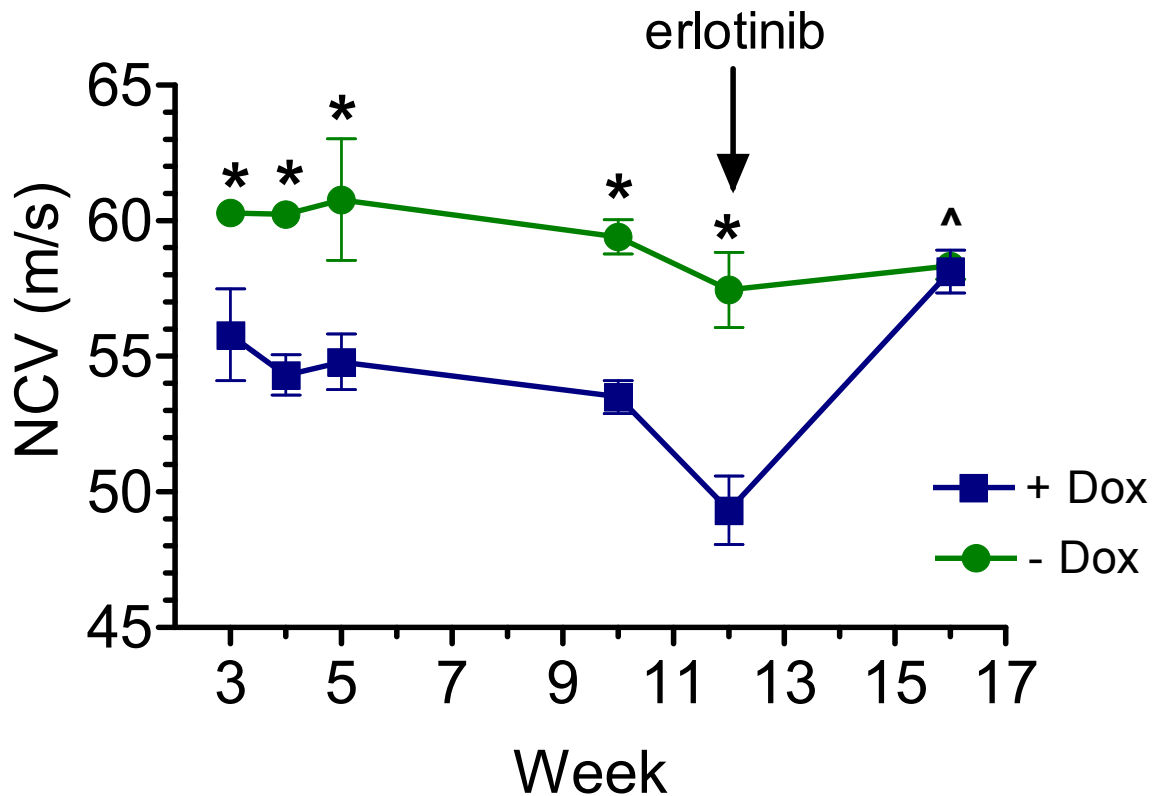


Figure 3.25: Motor nerve conduction velocity in $P_0rtTA \times caErbB2$ mice treated with doxycycline (Dox). Mice treated with Dox developed a significant deficit in MNCV after three weeks of Dox treatment. After 12 weeks of Dox treatment mice were treated with 25 mg/kg erlotinib, which completely restored MNCV. * = $p < 0.05$ for + Dox vs. - Dox. ^ = $p < 0.05$ for MNCV vs. + Dox at week 12.

Since the expression of constitutively active ErbB2 is limited to Schwann cells, and thus myelinated nerves, we wanted to determine whether or not the deficits induced by increased ErbB2 activation were the result of demyelination. To address this issue we quantified the g-ratio in sciatic and tibial nerves from doxycycline treated and untreated $P_0rtTA \times caErbB2$ mice. We quantified the g-ratio using the traditional method and

CellProfiler software because the traditional method is time consuming, tedious, and susceptible to inaccuracies. Using the traditional method (Figure 3.26) and CellProfiler (Figure 3.27) we did not observe any differences between doxycycline treated and control P₀rtTA x caErbB2 mice. These results suggest that the deficits in mechanical sensitivity and

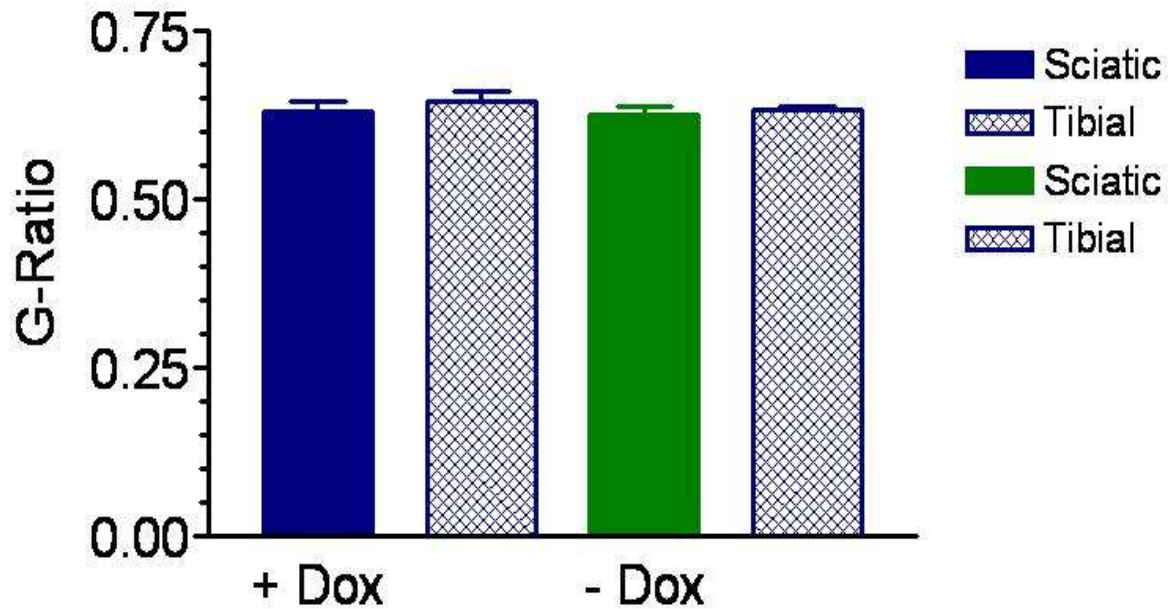


Figure 3.26: g-ratio quantification using the traditional method in doxycycline (Dox) treated and untreated control P₀rtTA x caErbB2 mice. The induction of constitutively active ErbB2 expression using Dox does not result in changes in myelin thickness as measured by quantification of the g-ratio.

MNCV mediated by the over-activation of ErbB2 are not the result of demyelination. These results do not rule out the possibility that longer periods of ErbB2 activation contribute to the development of neuropathy, but for the time course studied our results do not imply that

neuropathy arises from demyelination.

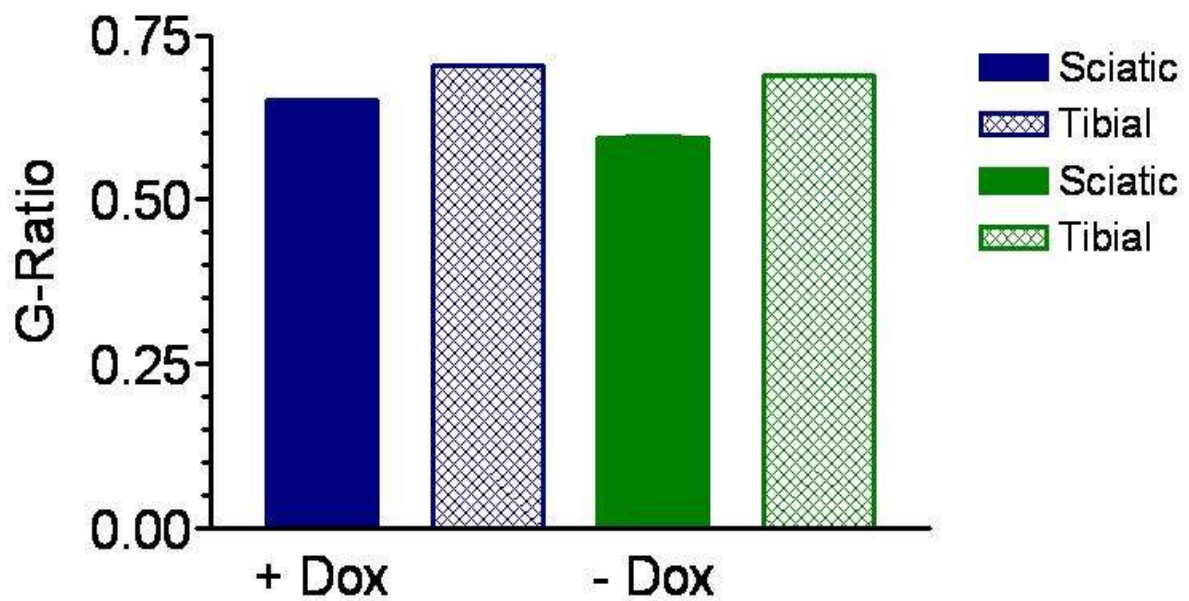


Figure 3.27: g-ratio quantification using CellProfiler in doxycycline (Dox) treated and untreated control P_{0rtTA} x $caErbB2$ mice. The induction of constitutively active ErbB2 expression using Dox does not result in changes in myelin thickness as measured by quantification of the g-ratio.

CHAPTER 4: DISCUSSION

4.1 A Role for Cav-1 in Regulating Neuregulin Signaling in Schwann Cells

Caveolin-1 plays essential roles in endocytosis, signal transduction, and cholesterol homeostasis and has been implicated in numerous pathological processes including several human cancers, diabetes, vascular diseases, lipid storage abnormalities, and ageing (Park, Cohen et al. 2003; Cohen, Hnasko et al. 2004). The viability and fertility of Caveolin-1 knockout mice demonstrates that Cav-1 is not necessary for life, but the increased susceptibility to metabolic and tissue abnormalities highlight the importance of Cav-1 in many cellular processes. Indeed, Cav-1^{-/-} mice are prone to neurological abnormalities, vascular problems, and adipocyte dysfunction (Razani, Engelman et al. 2001; Razani, Combs et al. 2002; Trushina, Du Charme et al. 2006). We have shown that absence of Cav-1 corresponds with an earlier onset and development of a more severe diabetic neuropathy. Mice lacking Cav-1 demonstrate significant nerve dysfunction behaviorally and electrophysiological by one week of hyperglycemia. Although Cav-1 deficient mice are prone to vascular problems which can contribute to the development of neuropathy, it is unlikely that the sensory deficits are because of such vascular abnormalities since non-diabetic Cav-1^{-/-} mice demonstrated similar characteristics to wild-type mice. Caveolin-1 is also known to serve as a scaffolding protein in membranes and could therefore play a significant role in the structural organization of the myelin sheath and axonal membranes (Silva, Maldonado et al. 2007). However, this scaffolding function of Cav-1 is not necessary for myelination since the peripheral nerves of adult Cav-1^{-/-} mice are not morphologically

different from wild-type mice. Furthermore, Cav-1^{-/-} mice demonstrate similar sensitivity to thermal and mechanical stimulation and nerve conduction velocities as wild-type mice.

One of the major functions of Cav-1 is to regulate signal transduction pathways. There is considerable evidence showing Cav-1 is involved in insulin signaling, particularly in acting as a positive regulator of insulin signaling by stabilizing the insulin receptor (Goldberg, Smith et al. 1987; Goldberg, Smith et al. 1987; Yamamoto, Toya et al. 1998; Gustavsson, Parpal et al. 1999; Nystrom, Chen et al. 1999; Cohen, Combs et al. 2003; Cohen, Razani et al. 2003). However, in the majority of its interactions with tyrosine kinases and heterotrimeric G-proteins, Cav-1 acts as a negative regulator of signaling (Sargiacomo, Sudol et al. 1993; Lisanti, Scherer et al. 1994; Li, Okamoto et al. 1995; Cohen, Hnasko et al. 2004). The data we have gathered suggests that Cav-1 is an endogenous negative regulator of ErbB2 activity. In an effort to clarify whether the hyperglycemia-induced downregulation of Caveolin-1 in kidney and nerve tissue contributed to the pathophysiological development of DPN we evaluated nerve function in the absence of Cav-1 (Tan, Rouen et al. 2003; Komers, Schutzer et al. 2006). In doing so, we observed an earlier, more severe development of nerve dysfunction in diabetic Cav-1^{-/-} mice that correlated with an increase in ErbB2 activation. This excess ErbB2 activation contributed to the development of nerve dysfunction since inhibition of ErbB2 activity with PKI 166 and erlotinib restored nerve function. The observation that diabetic wild-type mice recovered from nerve dysfunction following pharmacologic inhibition of ErbB2 suggests that receptor over-activation contributes to the development of nerve dysfunction. However, Cav-1^{-/-} mice consistently developed significantly more severe deficits in mechanical sensitivity and MNCV. That these same mice also fully recovered from nerve dysfunction with treatment with PKI 166 and erlotinib

not only suggests that ErbB2 activation contributes to the pathophysiological development of DPN, but that Cav-1 is also able to modulate ErbB2 activity.

The mechanism by which Cav-1 modulates ErbB2 activity is presently unknown, but there are two distinct possibilities. First, Cav-1 may interact directly with ErbB2 at the plasma membrane, prevent it from interacting with ErbB3, and thereby prevent its activation. The dimerization and scaffolding domains of Caveolin-1 allow it to function in the compartmentalization of caveolae and prevent the unnecessary interaction of receptors and signaling molecules (Lisanti, Scherer et al. 1994; Dobrowsky, Rouen et al. 2005). Cohen and colleagues have proposed that the ability of Cav-1 to act as a scaffolding protein and to regulate receptors and signaling molecules allows it to control signal transduction by sequestering signaling agents in caveolae (Cohen, Hnasko et al. 2004). Our data indicate that Cav-1 may be an endogenous regulator of ErbB2 and that its absence modifies ErbB2 activity. It is possible that under normal conditions Cav-1 interacts with ErbB receptors and prevents unnecessary dimerization. However, in the absence of Caveolin-1 receptor interaction and dimerization could increase significantly (See Figure 4.1). Although our data suggest that Cav-1 serves as an endogenous regulator of ErbB2 activity, its expression does not impact basal ErbB2 activity since we did not observe any differences in mechanical sensitivity or MNCV between non-diabetic Cav-1^{-/-} and wild-type mice. This inconsistency allows for a second possible mechanism by which Cav-1 modulates ErbB2 activity, it may mediate changes in the trafficking or localization of ErbB2. Most tyrosine kinase receptors are internalized and degraded or recycled in a ligand dependent manner (Wiley 2003). Since ErbB2 lacks a ligand-binding domain, it is internalized in a ligand independent manner. Additional evidence demonstrates that ErbB2 is internalized at a rate too slow to be regulated

in a ligand dependent manner (Baulida and Carpenter 1997). ErbB2 – ErbB3 heterodimers are likely internalized via a clathrin independent endosomal pathway (Wiley 2003).

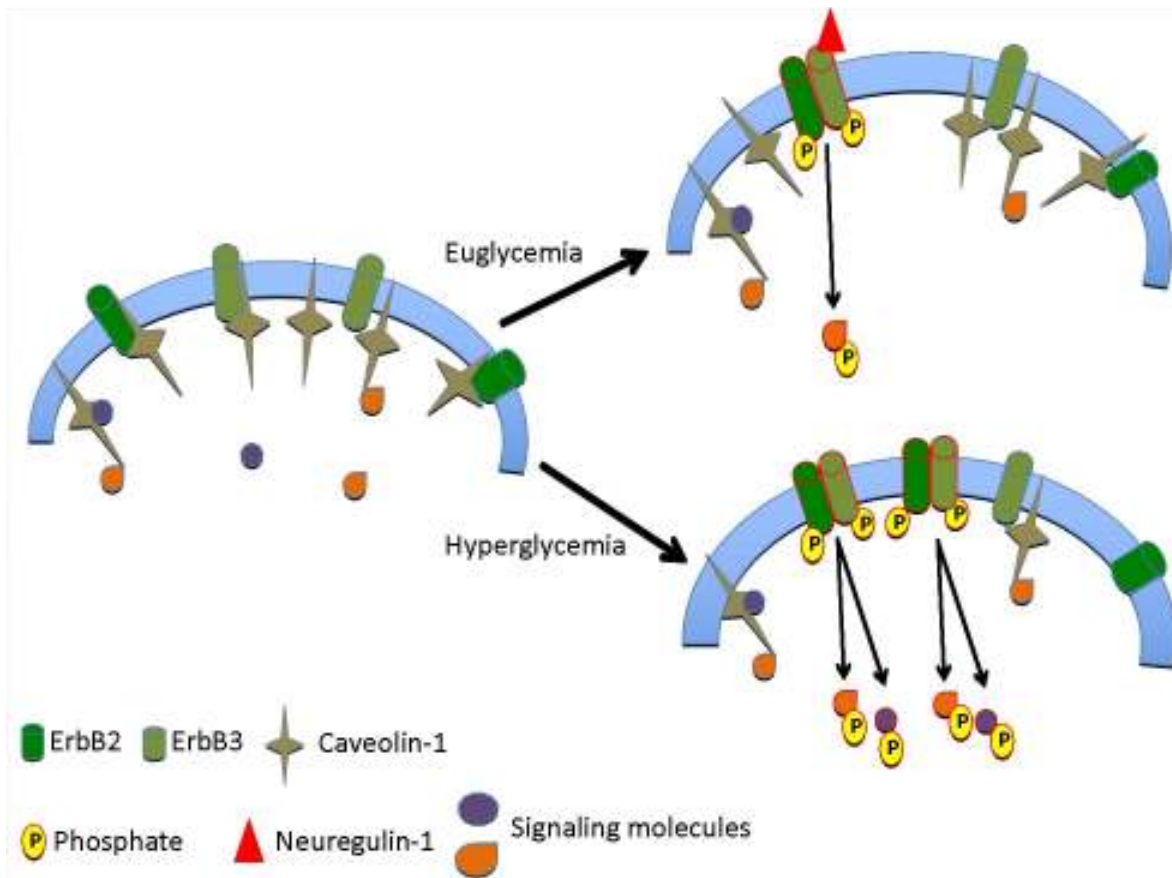


Figure 4.1: Proposed mechanism by which hyperglycemia and the absence of Caveolin-1 result in increased ErbB2 activation. Under normal, euglycemic conditions, the binding of Neuregulin-1 to the ErbB3 receptor induces dimerization and cross-phosphorylation with ErbB2. Activation of the dimer then activates signaling molecules. However, during hyperglycemic conditions, excess glucose combined with the absence of Caveolin-1 could lead to increased dimerization and receptor activation in the absence of a ligand. Since Caveolin-1 functions as a scaffolding protein, the loss of this function could allow for increased receptor-receptor interactions and thus, dimerization.

Once internalized, the receptors are distributed to endosomes and degraded or recycled back to the plasma membrane. The ErbB2 and ErbB3 receptors have a relatively short lifespan and are internalized and recycled several times during their lifespan (Waterman, Sabanai et al. 1998; Citri, Alroy et al. 2002; Wiley 2003). An aberration in this process could significantly alter ErbB2 signaling. Whether or not ErbB2 internalization or trafficking

requires Cav-1 has yet to be determined. However, since Cav-1 plays a major role in clathrin independent endocytosis and intracellular trafficking, it is likely to be involved in the internalization and trafficking of ErbB2.

Whether the mechanism by which Cav-1 modulates ErbB2 activity is by altering Cav-1's ability to regulate signaling events, by modifying receptor internalization and trafficking, a mixture of the two, or some other process, our data suggest that hyperglycemia significantly exacerbates these affects. Since Cav-1 expression does not influence basal ErbB2 activity, hyperglycemia is a critical component of the changes in ErbB2 activity that we observed. Although we have shown the absence of Cav-1 results in more rapid development of DPN and correlates with a significant increase in the activation of ErbB2, much remains unknown.

One surprising finding from our study of hyperglycemia in Cav-1^{-/-} mice was the prompt development of thermal insensitivity. Diabetic wild-type mice gradually develop thermal insensitivity, but it is much delayed compared to diabetic Cav-1^{-/-} mice. We did not observe any deficits in SNCV up to 12 weeks of diabetes, therefore thermal insensitivity cannot be attributed to impaired SNCV. Additionally, we did not observe a significant loss of epidermal nerve fibers from diabetic Cav-1^{-/-} mice. In light of these observations, delayed paw withdrawal can then be explained by the observed dysfunction of motor nerves. Diabetic Cav-1^{-/-} mice demonstrate significant slowing of MNCV. However, if this were the case, we would have expected to see some improvement in thermal sensitivity with ErbB2 inhibition. We did not observe a recovery from thermal insensitivity after treatment with PKI 166 or erlotinib. This contradiction can be reconciled by the possibility that the rapid development of thermal insensitivity in diabetic Cav-1^{-/-} mice is a result of some metabolic

difference between genotypes. Caveolin-1^{-/-} mice are known to have problems significant metabolic problems (Razani, Combs et al. 2002). The loss of thermal sensation in the absence of deficits in SNCV combined with the inability of PKI 166 and erlotinib to improve thermal sensitivity makes this an intriguing problem that needs further attention.

4.2 ErbB2 Activation in the Diabetic Nerve

The complex assortment of signaling networks influenced by the neuregulin-1/ErbB2 ligand/receptor pair are critical for the survival, growth, and differentiation of Schwann cells (Syroid, Maycox et al. 1996; Ogata, Iijima et al. 2004; Monje, Athauda et al. 2008). Pathological activation of these signaling networks may promote demyelination and the development of a neuropathic phenotype independent of hyperglycemia. It has been shown that increased signaling through the ras/raf/ERK pathway mediated by ErbB2/ErbB3 activation induced demyelination and dedifferentiation of Schwann cells (Zanazzi, Einheber et al. 2001; Harrisingh, Perez-Nadales et al. 2004). Further evidence suggests that disruption of ErbB2 signaling in adult Schwann cells leads Schwann cell dedifferentiation and progressive sensory loss (Chen, Rio et al. 2003; Huijbregts, Roth et al. 2003). Additionally, Tapinos *et al.* has shown that an increase in ErbB2 activity induced by leprosy bacilli mediates early events that result in demyelination (Tapinos, Ohnishi et al. 2006). Our findings broaden these observations and provide the first recognition that ErbB2 activation is a contributing factor in the pathophysiological development of DPN.

The development of deficits in thermal sensitivity, mechanical sensitivity, and MNCV correlated with an increase in ErbB2 activity in diabetic Cav-1^{-/-} and wild-type mice. As early as one week after the induction of hyperglycemia, Cav-1^{-/-} mice developed

significant MNCV slowing and mechanical and thermal insensitivity. Diabetic wild-type mice, although delayed compared to Cav-1^{-/-} mice, also developed significant deficits in these same indices of DPN. During initial periods of DPN development, when mice are becoming insensitive to thermal and mechanical stimulation and demonstrating deficits in MNCV, diabetic mice demonstrate significantly increased activation of ErbB2.

Pharmacological inhibition of ErbB2 activation promotes the recovery from deficits in mechanical sensitivity and MNCV. Both ErbB2 inhibitors that we used are not ErbB2 specific and therefore could inhibit EGFR and ErbB4 as well. However, it is unlikely that inhibition of these receptors contributes to the recovery we observed because these receptors are absent or expressed at low levels in adult Schwann cells (Grinspan, Marchionni et al. 1996; Atanasoski, Scherer et al. 2006). Taken together these data suggest that acute hyperglycemia produces neuropathy primarily through increased activation of ErbB2. Early events in the development of neuropathy after acute hyperglycemia can be attributable to glucotoxicity, but whether or not increased ErbB2 activation is result of glucotoxicity is not known (Tomlinson and Gardiner 2008). The data we obtained from chronically diabetic mice argues against such a suggestion.

The data we have obtained from studies of chronic hyperglycemia suggest that multiple factors contribute to the development of DPN. After 12 weeks of diabetes, treatment with the ErbB2 inhibitor erlotinib for three weeks only partially improves deficits in mechanical sensitivity and MNCV in Cav-1^{-/-} and wild-type mice. Similar results are obtained after 20 weeks of diabetes and 6 weeks of erlotinib treatment. Diabetic mice demonstrate a partial recovery from deficits in mechanical sensitivity and MNCV. It is important to note that we did observe a significant decline in SNCV in this study, but

erlotinib treatment had no significant affect in improving thermal insensitivity or SNCV. The partial recovery in mechanical insensitivity and MNCV deficits that we observed in Cav-1^{-/-} and wild-type mice after long periods of diabetes suggests that pathological activation of ErbB2 is not the sole contributing factor to the maintenance of nerve dysfunction and DPN. It is likely that metabolic changes associated with hyperglycemia make significant contributions as well. These metabolic changes have been discussed previously and include, increased flux through the polyol and hexosamine pathways, increased activation of PKC, and increased formation of AGEs. Inhibiting the accumulation of byproducts of these pathways or inhibiting the pathways themselves restores some nerve function in experimental diabetes (McLean, Pekiner et al. 1992; Mizuno, Kato et al. 1999; Nakamura, Kato et al. 1999; Takeuchi, Bucala et al. 2000; Toth, Rong et al. 2008). Our data, combined with that from various other groups, suggest that chronic hyperglycemia produces a neuropathic phenotype in multiple ways.

The data we gathered from our double transgenic mice expressing a constitutively active form of ErbB2 when treated with doxycycline support our hypothesis that ErbB2 activation alone contributes to the development and maintenance of DPN. Normoglycemic mice treated with doxycycline demonstrate significant deficits in mechanical sensitivity and MNCV. When doxycycline is removed, deficits in mechanical sensitivity disappear. Furthermore, inhibition of ErbB2 activation with PKI 166 or erlotinib also restores normal mechanical sensitivity and MNCV. Again, it is important to note that the restorative affects of PKI 166 and erlotinib are likely not because of EGFR and ErbB4 inhibition since the expression of these receptors is insubstantial in adult Schwann cells (Grinspan, Marchionni et al. 1996; Atanasoski, Scherer et al. 2006). Additionally, the restorative affects of these

inhibitors are not likely to be because of the transgenic status of these mice since the inhibitors did not have an affect on mice not receiving doxycycline but still receiving drug. These data confirm that activation of ErbB2 in the absence of hyperglycemia is sufficient to induce nerve dysfunction.

4.3 Effects of pathological ErbB2 activation

The data we have gathered suggests that pathological ErbB2 activation predominantly affects myelinated nerves. Diabetic Cav-1^{-/-} and wild-type mice develop sustained deficits in mechanical and thermal sensitivity and MNCV. Evaluating mechanical sensitivity indirectly assesses the function of myelinated A-δ fibers (Julius and Basbaum 2001; Calcutt 2002). Although a small portion of A-δ fibers function in the propagation of information regarding thermal sensation, we used a protocol designed to limit the activation of A-δ fibers (Yeomans and Proudfit 1996). That diabetic mice develop significant mechanical insensitivity and MNCV deficits suggest that myelinated fibers are rapidly affected by hyperglycemia. The development of nerve dysfunction correlates with increased ErbB2 activation. We have shown that inhibition of ErbB2 activity restores normal mechanical sensitivity and MNCV after a short duration of diabetes and partially after longer periods of diabetes. Additionally, treatment with PKI 166 and erlotinib has no affect on thermal sensitivity or SNCV at the time points we tested. These observations imply that ErbB2 activation in the Schwann cells of myelinated nerves contributes to the development of mechanical insensitivity and slowed MNCV

Although our data suggest that mechanical insensitivity and MNCV deficits are attributable to increased ErbB2 activation in myelinated nerves, it is not likely that these

deficits arise as a result of demyelination. The compact myelin protein P₀ comprises approximately 50% of the myelin sheath and a loss of this protein results in nerve dysfunction (Owens and Boyd 1991; Giese, Martini et al. 1992). However, after a short period of hyperglycemia we did not observe a change in the expression of P₀. Furthermore, we did not observe an increase in the g-ratio in chronically diabetic mice or our double transgenic mice. The optimal g-ratio is ~0.65 and an increase in the g-ratio is indicative of thinner myelin sheaths and demyelination (Waxman 1980). Demyelination has been shown in mice that were diabetic for nine months, but we did not observe changes in our mice after six months (Kennedy and Zochodne 2005). It is possible that the traditional method of quantifying g-ratio using ImageJ is not sensitive enough to distinguish minor changes in g-ratio. However, when using CellProfiler to semi-automate the process of quantifying large numbers of axons, although more sensitive, we did not observe any differences in g-ratio between mice treated with doxycycline and mice not treated with doxycycline. It is possible that demyelination would develop in our mice if our studies were extended for an additional two to four months. However, at the duration of diabetes that we studied, we cannot attribute the development of mechanical insensitivity and MNCV deficits to demyelination.

Demyelination is not the only change that could lead to slowed MNCV and deficits in mechanical sensitivity. Modified ErbB2 signaling could produce changes in one of several signaling networks in dorsal root ganglion (DRG) neurons could dramatically alter nerve function. Indeed, activation of p42/p44 mitogen activated protein kinase (MAPK) signaling has been implicated in demyelination (Harrisingh, Perez-Nadales et al. 2004; Tapinos, Ohnishi et al. 2006). Furthermore, STZ induced diabetes has been shown to enhance p42/p44 MAPK signaling in DRG neurons, but not in sural nerve (Fernyhough, Gallagher et

al. 1999; Purves, Middlemas et al. 2001). Although, ErbB2 activation may increase in DRG neurons, whether this contributes to DPN requires additional work.

Although we have not observed demyelination in diabetic or doxycycline treated double transgenic mice, nerve conduction velocity is associated with axon diameter, myelin thickness, which is proportional to internode length (Waxman 1980; Voyvodic 1989). These studies imply that changes in Schwann cell morphology could impact nerve conduction velocity. For instance, changes in paranodal morphology, nodal length, or internodal length could disrupt salutatory conduction and result in slowed nerve conduction velocity. Whether or not changes in Schwann cell or nodal morphology have a detrimental affect on nerve function has not been studied. However, we have observed the localization of activated ErbB2 in the paranodal region of sciatic nerves in diabetic mice (See Figure 4.2). Again, we have not examined whether or not diabetes produces significant changes in localization of ErbB2 activity or whether there are differences in paranodal activation of ErbB2 between diabetic and non-diabetic mice. We have shown though that changes in ErbB2 activity does increase in whole nerve lysates from diabetic mice and that this increases correlate with nerve dysfunction. It is therefore plausible that the increase in ErbB2 activity that we observed is at least partially confined to the paranodal region.

Hyperglycemia induced activation of ErbB2 predominantly impacts myelinated nerves, but we have also obtained data showing that increased ErbB2 activity impacts innervation of the planta pedis. Although other groups have reported denervation as early as four weeks after induction of diabetes, we did not detect a significant loss of nerve fibers in the epidermis until much later (Beiswenger, Calcutt et al. 2008; Johnson, Ryals et al. 2008).

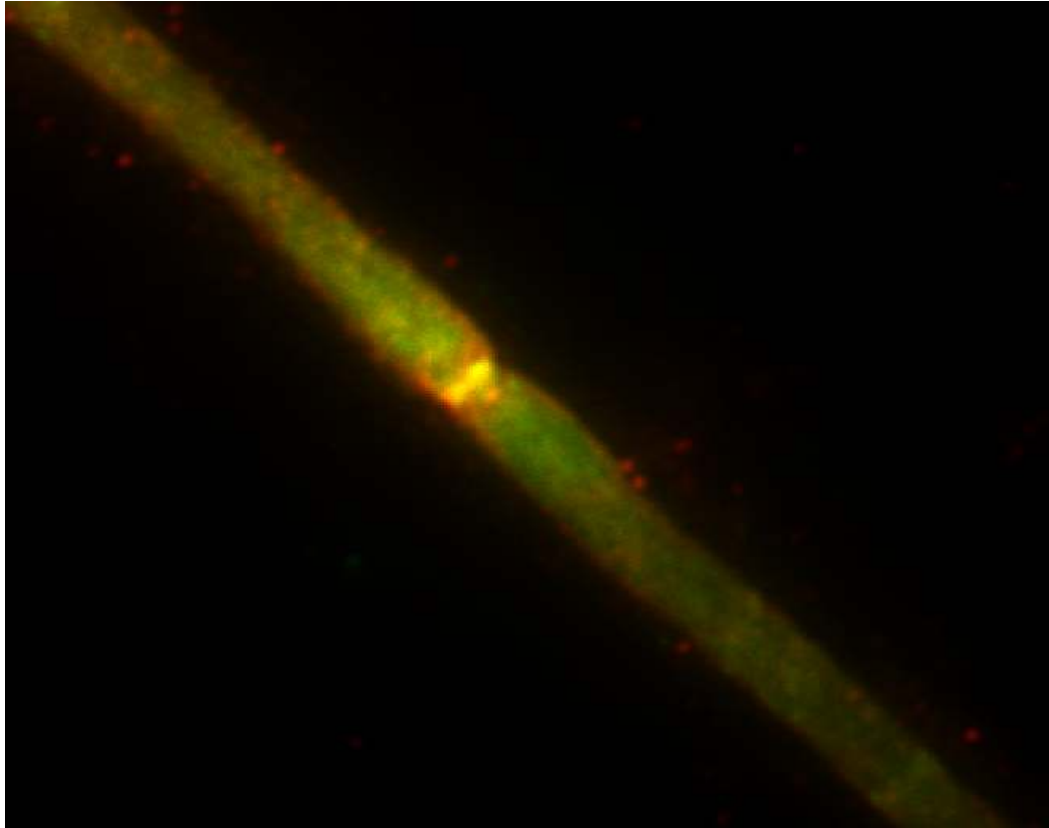


Figure 4.2: Localization of pErbB2 to paranodal region of sciatic nerve. Teased sciatic nerve was stained with E-cadherin (Red) and pErbB2 (green) and imaged with a 60X objective. E-cadherin is a marker of the paranodal region and staining shows that pErbB2 is present in this region as well.

After 26 weeks of hyperglycemia, we observed a significant decline in the number of nerve fibers in the epidermis. The reason for detecting fiber loss at different time periods can be explained by the use of different methodologies, particularly STZ administration and mouse strains. With the length of time required to identify the loss of fibers it could be said that metabolic changes associated with chronic hyperglycemia, and not ErbB2 activation, were responsible for the loss of fibers. However, treatment with erlotinib prevented the reduction in epidermal innervation. This finding was surprising since ErbB2 expression is limited in the unmyelinated nerves of the epidermis. Therefore, there should only be minor increases in ErbB2 activity in this population of cells. It is important to note that the ability of erlotinib to

prevent the loss of epidermal fibers in diabetic mice cannot be explained by drug treatment since non-diabetic mice treated with erlotinib do not demonstrate an increase in epidermal fibers. One possible explanation for this mystery is increased ErbB2 activity in DRG neurons. It is plausible that increased ErbB2 activity in DRG neurons could lead to loss of distal nerve fibers, but whether or not this is the case is not known. Furthermore, although we observed re-innervation of the epidermis with erlotinib treatment, we did not see an improvement in thermal sensitivity. There was a trend toward increased sensitivity with erlotinib treatment, suggesting longer duration of treatment or increased doses may have an impact on thermal sensitivity. It may be that re-innervation is occurring, but the degree of re-innervation is not sufficient to allow functional recovery. How ErbB2 activity mediates denervation in the epidermis is an interesting question raised by our studies and needs further attention.

From the data that we have gathered, it seems that increased ErbB2 activation is modifying two distinct populations of cells. First, increased ErbB2 in Schwann cells produces mechanical insensitivity and MNCV deficits. The efficacy of PKI 166 and erlotinib to restore deficits in mechanical sensitivity and MNCV in diabetic Cav-1^{-/-} mice, diabetic wild-type mice, and our double transgenic mice underscores the importance of ErbB2 activation in the development of DPN. Second, increased ErbB2 activity over an extended period of time may contribute to epidermal denervation, possibly by acting on axons or DRG neuron cell bodies. Again, the efficacy of erlotinib to prevent fiber loss highlights the role of increased ErbB2 activity in the development of DPN.

4.4 Conclusions

In summary, the data we have obtained provide the primary evidence suggesting that altered neuregulinism induced by hyperglycemia is a pathological process that contributes to the development and maintenance of DPN. Increased ErbB2 activation correlates with the development of mechanical and thermal insensitivity, deficits in MNCV, and epidermal denervation. The mechanism or mechanisms by which increased ErbB2 activation mediates pathological changes is unknown. Mechanical insensitivity and reduced MNCV cannot be explained by demyelination, but the potential of more subtle morphological changes in Schwann cells needs to be studied. Additionally, the mechanism responsible for the loss of epidermal fibers is unknown but may involve changes in ErbB2 mediated signaling in DRG neurons. Both cases make clear that changes in ErbB2 activity have clear functional and morphological consequences. ErbB2 is known to interact with several signaling molecules and can contribute to the activation of several signaling networks. Pathological activation of ErbB2 may alter signaling in one of these pathways. Indeed, recent data suggest that an increase in p38 MAPK activity may be involved since inhibition of p38 MAPK improves nerve conduction velocity deficits (Price, Agthong et al. 2004). Furthermore, ErbB2 may increase p38 activity (Kim, Yong et al. 2009). There are likely other mechanisms by which ErbB2 could mediate the changes we have observed and studying these mechanisms may provide novel therapeutic approaches for treating DPN.

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